STRUCTURE AND FUNCTION OF THE BULLA, SECOND MAXILLAE AND MAXILLARY DUCTS OF *SALMINCOLA CALIFORNIENSIS* (DANA, 1852) (COPEPODA: LERNAEOPODIDAE)

by

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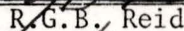

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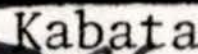

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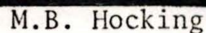

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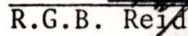
SUPERVISOR: Dr. R.G.B. Reid



ABSTRACT

Structural details of the bulla, second maxillae, and maxillary duct system of adult female Salmincola californiensis (Copepoda: Lernaeopodidae) are considered in relation to their co-operative primary function of uptake of dissolved substances directly from the tissue fluids of the host, and some biological implications of this previously undescribed function are discussed. The mechanisms of haemal fluid circulation in S. californiensis are described briefly in relation to the maxillary uptake system. A technique is described for maintaining S. californiensis in vitro with the bulla isolated from the medium surrounding the body of the copepod, for periods of 14 to 21 days. Using this technique the relative permeability of the bulla, maxillary duct walls and external cuticle surfaces to a variety of types of dissolved substances was investigated. The external cuticle was found to be generally impermeable to most ionized readily water-soluble substances tested, including $KMnO_4$ in saturated solution, but permeable to some relatively small and weakly polar organic molecules of low water solubility. The maxillary ducts were found to be permeable to a variety of types of ions and polar molecules ranging in molecular weight up to at least 350 (including ionic vital stains, inorganic ions, some $^{14}C$-amino acids and $^{14}C$-glucose), but were relatively impermeable to un-ionized weakly polar organic molecules. Five potential chemotherapeutic agents (Ronnel, Trolene 20L, Ruelene, Ruelene 25E, and phenoxethol) were screened in vitro for toxicity to S. californiensis and those showing potential control ability were further tested in vivo on infected sockeye (Oncorhynchus nerka) smolts. A successful eradication procedure has not yet been developed as those compounds showing toxicity to Salmincola (Ruelene and phenoxethol) were more toxic to infected fish. However, a testing procedure is outlined and recommendations are made for future work of this nature. In addition, an hypothesis

proposing a possible sequence of evolution and development of the bulla and maxillary duct system is presented and implications concerning the origin of family Lernaeopodidae are briefly discussed.



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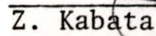

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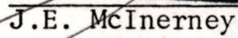

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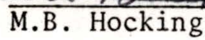

M.B. Hocking

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# INTRODUCTION

The Lernaeopodidae have long been known as external parasitic copepods of fishes, both in fresh and marine waters. However, taxonomy within the family has only recently been clarified, due primarily to numerous works by Kabata, and very little is yet known concerning the biology of these parasites. Members of this family are characterized by a unique structure, the "attachment organ" or bulla, a single unpaired acellular structure of variable size, shape and consistency, which joins the tips of the second maxillae and is embedded in the surface tissues of the host, thereby effecting permanent anchoring of the adult female copepod at a fixed site on the external surface of the host fish. The bulla is formed in a collapsed state within the frontal region of the cephalothorax of the copepod, prior to the final moult to the adult female stage. It is implanted in the host's tissues, attached to the second maxillae and inflated at the time of the final moult, and thereafter persists throughout the adult life of the copepod in all members of the family, with the single exception of a small group of aberrant forms parasitic on skates and rays. In the latter the bulla is present during early stages of adult development and provides temporary attachment, but is later superseded by either a simple anchor-like or a more complex dendritic structure which develops from the tips of the second maxillae (Kabata and Cousens, 1972;1973).

The origin and possible functions of the bulla have long puzzled researchers studying members of this family and numerous hypotheses have been advanced, generally from investigation of preserved and often incomplete or mutilated specimens. Its origin was first correctly interpreted by Friend (1941) and recently confirmed by Kabata and Cousens (1973), both studies involving observation of fresh and living material. However, neither the bulla nor its

associated ducts and glandular tissue within the second maxillae have to date been described or studied in detail. Neither have any explanations of their possible functions ever been demonstrated, other than the obvious one of attachment to the host. Hence any intelligent speculation concerning the possible advantages of these unique structures has previously been impossible.

Recent work (Kabata and Cousens 1972, '73, '77) has strongly suggested a transport function for this system, at least in the primitive fresh-water branch of the family consisting of Salmincola and its close relatives. If this is the case, the position of the Salmincola group within family Lernaeopodidae as that group most closely allied to the ancestral form might well permit inferences as to the original function of this system, and perhaps also concerning the origin of the Lernaeopodidae and their abandoning of the seeming advantage of mobility in favour of a sessile existence. On a more practical level, an understanding of the functioning of any exchange processes occurring via the bulla and its ducts might well provide valuable clues to the biology of Salmincola and its relatives which could be most useful in designing effective control measures or eradication procedures for these sometimes troublesome and as yet uncontrollable pests of fresh-water salmon and trout in hatcheries and rearing facilities.

This study describes in detail the structure of the bulla and the ducts of the second maxillae of Salmincola californiensis, and demonstrates some functions of these structures in passage of materials in dissolved form from host to parasite tissue fluids. An explanation is advanced for the observed resistance of Salmincola to all usual control methods generally found effective for many "typical" ectoparasites of salmonid and other aquaculture fish stocks, and some recommendations are offered concerning development of potential

control measures. Finally, an argument is presented for the origin and development of the bulla and duct system as it occurs in *Salmincola californiensis*, and hence for the origin and success of the Lernaeopodidae.

## SUMMARY OF THE LITERATURE

Systematics in the genus Salmincola have recently been reworked by Kabata (1969), who recognized 15 distinct species, their combined distribution being circumpolar in the northern hemisphere. Kabata divides the genus into two subgenera Salmincola (Salmincola), which is parasitic almost entirely on salmonid fishes (Salmonidae), and Salmincola (Brevibrachia ), which occurs exclusively on coregonid fishes (Coregonidae). The genus normally occurs and reproduces only in fresh waters of rivers and lakes, though adult females of at least some species can survive (but not reproduce) in the ocean on anadromous fishes for an undetermined period of time consisting of at least several weeks and possibly several months (Kabata - personal communication).

Salmincola californiensis (Dana, 1852) is a Pacific species occurring both in Asia and North America (Kabata, 1969), where it is often a serious problem in fresh-water salmon and trout hatcheries and fish-farms. While low-level infections encountered in the wild often appear of little consequence, the extremely high infection levels encountered as a result of crowding in hatchery conditions are eventually fatal, either directly due to inability to tolerate any slight additional stress, or indirectly due to secondary infection by fungi or bacteria. As there is at present no known control or eradication procedure for this parasite, its appearance in a hatchery generally implies eventual destruction and total loss of the infected stocks (Kabata - personal communication). Under similar conditions, S. californiensis has been shown to reduce egg yield from rainbow trout spawners, and possibly also viability, vigour and post-hatch survival of the trout hatchlings (Gall et al, 1972). Hence in at least one U.S. hatchery where rainbow trout adults are held for maturation and artificial spawning, Salmincola eradication by such drastic

and often impractical means as repeated chlorination of the hatchery and its headwaters to destroy all resident fish stocks acting as upstream sources of the infection, followed by restocking of the hatchery, has been carried out and considered justified in terms of increased production (Gall *et al*, 1972).

The life history and development of *Salmincola* was first proposed by Friend (1941) for *S. salmoneus*, using specimens collected from migrating *Salmo salar* in Scotland. The complete life-history, including several stages overlooked by Friend, was later established (Kabata and Cousens, 1973) using laboratory-cultured *S. californiensis* raised on sockeye salmon (*O. nerka*) experimental stocks.

The origin of the bulla or attachment organ was also first correctly interpreted and described by Friend (1941), this structure being produced and extruded from the frontal region of the cephalothorax of the chalimus IV female larva during the final moult to the adult stage. To effect permanent anchoring of the adult female the bulla was first implanted in a cavity eroded in the host's tissues, then attached to the tips of the post-moult second maxillae and inflated from its previously collapsed state to form the more familiar large mushroom-shaped "attachment organ" of the adult copepod. There then followed a period of rapid and extensive growth of the copepod parasite, without further moulting of the cuticle. These observations, initially thought somewhat fanciful and accepted with some reservation, were later confirmed and extended by Kabata and Cousens (1973), who described the entire attachment sequence in detail for *S. californiensis*.

A survey of the structure and histology of the bulla and surrounding host tissues at the attachment site of 24 species of parasitic copepods distributed

throughout the family Lernaeopodidae (Kabata and Cousens, 1972) showed the bulla to be a highly variable structure following three distinct lines of development within the family, in relation to the major groups of elasmobranch and teleost fishes infected. These results suggested that the form and function of the bulla might be related to the type of host and the conditions pertaining within the host's environment, or perhaps within its own surface tissues. More specifically, all species parasitic on marine fishes showed some form of reduction and often fusion of the second maxillae, and reduction of the bulla to a hardened, usually small structure of variable shape, often cemented into subdermal connective tissue of the host and apparently serving strictly for attachment. (Within the marine environment there were further distinct differences between the bullae and second maxillae of copepods parasitizing elasmobranchs and teleosts.) Conversely, all species occurring in fresh water on anadromous and fresh water fishes (a group consisting only of _Salmincola_ and closely related genera) possess large, well developed and usually contractile second maxillae which are completely separate (i.e. never fused together along all or part of their length) and a large, generally mushroom-shaped, unhardened and often elastic bulla. The latter is not cemented to the host's subdermal connective tissue in any way (with the exception of one aberrant genus, _Tracheliastes_, which cements its somewhat atypical bulla to the surface of a scale) and firmly attaches the copepod to the fish solely by nature of its mushroom or anchor-like shape being embedded in host tissues, which rapidly heal over it after implantation.

The position of _Salmincola_ and its allies within the family Lernaeopodidae as the most primitive existing group and the only group inhabiting fresh water may suggest a possible origin for the family in association with a fresh water

environment (Kabata and Cousens, 1972). It is likely that the Salmincola-type bulla may resemble that of ancestral lernaeopodids in form and function, since the hardened and reduced attachment structures of the more recently evolved marine representatives of the family follow a general trend toward loss of any other functions which may have originally been associated with attachment in fresh water, and a specialization solely for attachment to the surface of marine fishes.

A more recent study of bulla implantation and subsequent tissue damage in S. californiensis (Kabata and Cousens, 1977) reveals a close association between the bulla and the tissues of the host, with only a very thin reticulum of connective tissue covering the bulla and separating it from underlying tissues, which often include blood vessels and enlarged blood sinuses. Rarely was connective tissue encapsulation observed (in contrast to that commonly occurring with species infecting marine fishes) and there was virtually no subdermal tissue response to the presence of the bulla other than to the initial irritation and insults attending implantation. Observable tissue responses were usually restricted to an often profound epithelial proliferation in response to irritation at the surface caused by movements and feeding activities of the parasite. This unusual tolerance of a foreign structure by normally intolerant tissues, together with the complexity of the bulla and the ducts of the second maxillae, suggests a highly developed association between the copepod bulla and the tissues of the host; an association involving functions other than that of mere attachment, for which such a close and complex association seems unnecessary. It has thus been postulated that the bulla of S. californiensis may in addition to attachment also serve an exchange function between the body fluids of the copepod and the tissue fluids

of the host fish, though the nature of this proposed function is as yet undefined (Kabata and Cousens, 1972, 1977).

# METHODS

## I. Source of Experimental Material

Copepods used in this study were obtained from infected stocks of juvenile sockeye (*Oncorhynchus nerka*) maintained in the laboratory culture facilities of the Parasitology Section, Pacific Biological Station, Nanaimo, B.C. Fish were acquired from a hatchery, infected by exposure to already infected fish in the laboratory, and maintained in 300 gal. tanks of running fresh water at $10^{o}$-$15^{o}$C. The *Salmincola californiensis* infection was originally obtained from naturally infected fish stocks from Cultus Lake, B.C. Ripe egg-sacs were removed from the copepods, hatched artificially in small petri-dishes of dechlorinated water in a shaker-bath at $10^{o}$C., and the resulting copepodid larvae were used to infect healthy stocks of young sockeye from the hatchery, upon which the copepod infection was subsequently maintained and cultured. Periodically, groups of 20-50 fish were transported as needed to the Biology Department, University of Victoria, Victoria, B.C., and maintained in the dechlorinated fresh water aquarium system in 20 gal. tanks, for use in various experiments. Copepods were easily removed from freshly-killed fish using fine dissecting scissors and forceps, and after inspection under a dissecting microscope and rejection of deformed or damaged specimens, the remainder were rinsed in distilled water and retained for the *in vitro* experiments.

## II. Light Microscopy

Copepods were removed from the host, fixed in buffered formal-saline, dehydrated in an ethanol series, cleared in xylene and embedded in paraplast. 8μ serial sections were cut, mounted and stained with either a Mayer's haematoxylin, Celestine blue and Mallory's one-step triple-stain combination

(Humason, 1967), or a standard Mayer's haematoxylin and eosin series. In addition, a series of histochemical tests was performed on sections of the bulla in an effort to determine its general composition.

III. Scanning Electron Microscopy

Specimens were relaxed by gentle warming ($40^{o}$-$50^{o}$C), fixed overnight in buffered formal-saline, dissected with a sharp razor blade to expose the desired surface when necessary and rinsed in distilled water to remove any salts carried from the fixative which would otherwise form crystals during subsequent dehydration and drying. Specimens were dehydrated and dried by one of three methods:

(i) Quick-freezing in liquid nitrogen and freeze-drying at -$30^{o}$C.

(ii) Dehydration in acetone series, gradual replacement of the acetone with a liquid freon intermediate fluid (Genesolv D; electronic grade, Allied Chemical Co.) in an acetone/Genesolv D series using 10% increments of increasing Genesolv D concentration, and Freon critical point drying in a Bomar SP-900 Critical Point Dryer.

(iii) Quick-freezing, by dropping a small piece of tissue (in a drop of distilled water from a pipette) into acetone cooled to just above its freezing-point (-$94^{o}$C) in a test-tube immersed in liquid nitrogen, followed by freeze-substitution of the ice by acetone as the cooled acetone slowly warmed to room temperature ($1^{o}$-$2^{o}$C per minute, in a precooled styrofoam block). The acetone was then gradually replaced by liquid freon and the specimens were dried by the Freon critical point method, as in (ii) above.

While routine freeze-drying at -$30^{o}$C proved adequate for preparation of the bulla and external cuticular structures, Freon critical point

drying was used to check for freezing artifacts and for preparation of soft structures such as the thin cuticle and glandular tissues of the ducts in the second maxillae. The acetone freeze-substitution technique was developed to avoid distortion of the tissue due to shrinkage of the inner layers of the external thick cuticle during gradual stepwise dehydration in acetone at room temperature. The latter technique usually resulted in the inward curling of the free edges of the cut external cuticle, which often completely obscured the areas of interest. Dried specimens were coated with gold-palladium and either examined immediately in an ETEC Autoscan B-1 scanning electron microscope at 20 kv, or stored under vacuum for later study. All photographs were taken using Polaroid Type 55 positive/negative film.

## IV. Transmission Electron Microscopy

Fresh relaxed specimens were placed in a solution of 3% glutaraldehyde in 0.1 M cacodylate buffer at $4^oC$ and dissected in this solution on a wax plate over crushed ice. Small pieces of tissue were transferred to vials of this solution and fixation was continued on ice for 3 hours. Tissue pieces were rinsed in chilled 0.1 M sodium cacodylate buffer for 5 minutes, then post-fixed in 1.3% $OsO_4$ in 0.1 M sodium cacodylate buffer for an additional 3 hours at $4^oC$. Following a second rinse in 0.1 M buffer, the pieces of tissue were dehydrated in an acetone series and infiltrated and embedded in Epon 812 resin. All steps up to 90% acetone were carried out at $4^oC$.

Both thick ($1\mu$) and thin (grey to gold) sections were cut with glass knives on a Reichert U2 ultramicrotome. Thick sections were mounted on glass slides and stained with Richardson's Stain for light microscope study. Thin sections were mounted on Formvar-coated grids, stained with 2%

uranyl acetate and 0.2% lead citrate, and examined in a Phillips 300 Transmission electron microscope.

V. In vitro Isolation of the Bulla

Adult female S. californiensis were dissected from the fish, any adhering shreds of host tissue were removed from the bulla with fine forceps under a dissecting microscope, and any copepods with abnormally-shaped or damaged bullae were discarded, while intact specimens were transferred to a dish of fresh salmonid saline at 10°C. Specimens were then transferred, one at a time, to a dish of distilled water on the stage of a dissecting microscope and manipulated with fine forceps so that the bulla was inserted into the previously fire-polished tip of a hematocrit capillary tube. (Fire polishing in a small flame, as from an alcohol burner, was necessary to prevent the otherwise sharp edge of the tube bore from cutting through the cuticle of the copepod.) The bulla was firmly sealed in place by filling the space around the manubrium within the tip of the capillary tube with silicone stopcock grease, the latter being applied with a 5 cc plastic syringe carrying an 18 guage needle from which the bevelled tip had been removed. Distilled water drawn into the tube during this procedure was then withdrawn using a 5 cc plastic syringe fitted with a piece of fine teflon (PTFE) tubing, which easily entered the bore of the capillary and was cut to reach from the open end just to the subanchoral surface of the bulla within the opposite end. Another syringe similarly fitted with PTFE tubing was used to add to the tube above the bulla a small amount (50 μl) of salmonid saline or any other desired test solution (hereafter referred to as the "tube medium").

Finally, the capillary tube was inserted through a prepared cork and

placed in a one-dram glass vial containing three ml of distilled water (or other test solution, hereafter referred to as the "external medium"), such that the copepod at the lower end of the tube was submerged and suspended approximately one cm from the bottom of the vial (Fig. 1). Marking the level of fluid in the capillary tube facilitated detection of leaks at the stopcock grease seal, as did the inclusion of a small amount of a dye such as methylene blue or fluorescein in the tube medium as a tracer. It was found necessary to cut a small groove in the side of the cork to prevent buildup of pressure or vacuum in the vial during insertion or removal of the cork, which was otherwise a major cause of leaks at the stopcock grease seal about the bulla.

It should be noted that the use of distilled water rather than physiological saline during the mounting procedure is essential for two reasons:

(i) Adhesion of the stopcock grease to the bore of the capillary tube, which does not occur in physiological saline due to the interference of ions in solution and without which sealing of the tube around the bulla cannot be accomplished. Any tubes that leaked were discarded.

(ii) Detection of rupture or penetration of the cuticle of the copepod during handling, bulla insertion and sealing as indicated by body fluids leaking from the copepod, streams of which are readily visible in distilled water but easily overlooked in saline. Copepods damaged or suspected of being damaged during any stage of the mounting procedure were discarded.

Groups of 10 vials containing copepods mounted in this manner were accommodated in holes in small styrofoam blocks, to facilitate handling.

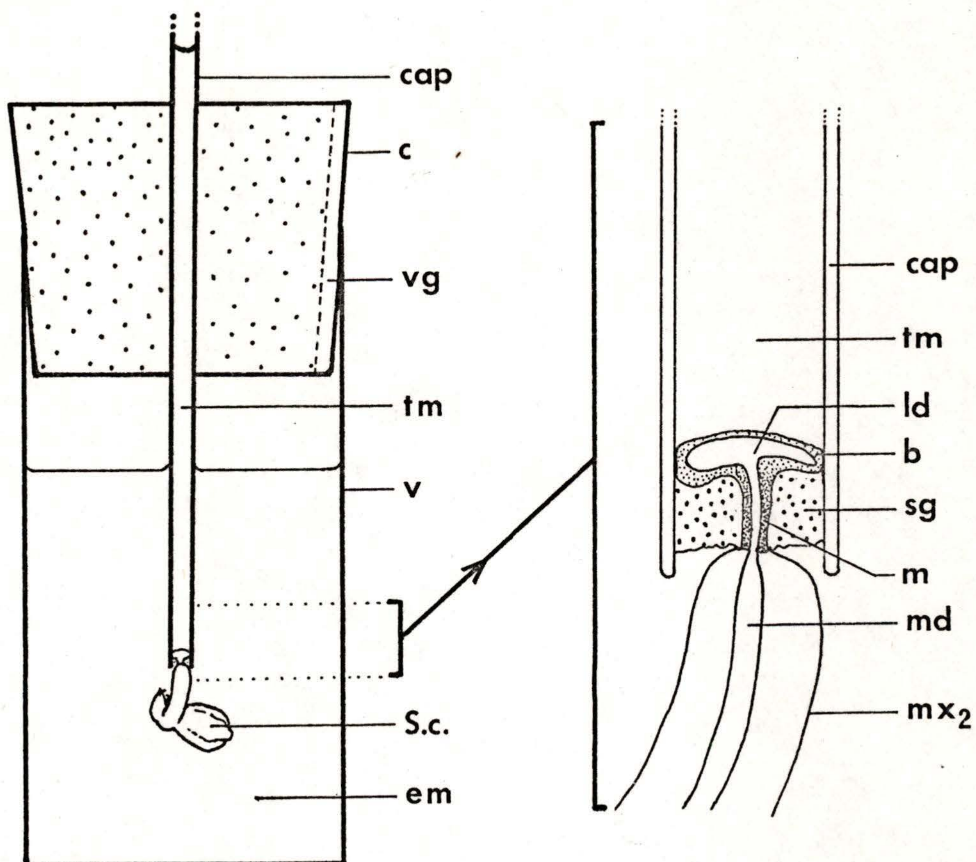



Fig. 1 Apparatus for mounting S. californiensis with bulla isolated from the surrounding external medium.

b - bulla; c - cork stopper; cap - hematocrit capillary tube; em - external medium in vial; ld - loop duct of bulla; m - manubrium of bulla; md - maxillary duct; $mx_2$ - second maxilla; S.c. - S. californiensis; sg - silicone stopcock grease seal; tm - capillary tube medium; v - 1-dram vial; vg - vent groove cut in cork stopper.

VI. Permeability Tests

After removal from the host, adult female S. californiensis were exposed in vitro to a variety of test solutions, in an effort to determine possible routes of entry into the parasite for these materials. Several methods of exposure were employed, as follows:

(i) Immersion - copepods were simply incubated in the test solution in standard Petri dishes at $10^{o}C$, with groups of 10 animals per dish.

(ii) Capillary tube isolation of the bulla - copepods were mounted in capillary tubes as previously described and placed in individual vials, each replicate consisting of a group of 10 animals. Test solutions were then presented either a) to the bulla alone, by being added to the capillary tube as the "tube medium", with distilled water as the "external medium" in the vial, or b) to the external body surface, excluding only the bulla, as the "external medium" in the vial, while salmonid saline (unless stated otherwise) was placed in the capillary tube as the "tube medium".

An experiment concerning a particular substance and route of entry consisted of at least two replicate test groups and a control group (when applicable), each containing ten copepods. Incubation during the experiments was routinely carried out at $10^{o}C$ in a refrigerator. The following solutions were tested:

(i) Ionic solutions

(a) $AgNO_3$; 0.01 M in distilled $H_2O$ (Croghan, 1958a)

(b) $KMnO_4$; saturated (approx. 0.5 M) in distilled $H_2O$ (Croghan 1958a)

(c) Salmonid physiological saline; (Hoar & Hickman, 1967, p. 262)

(d) $MgCl_2$; 0.1 M - 0.01 M

(ii) Metabolic inhibitors

(a) KCN; $10^{-2}$ M to $10^{-5}$ M (in tenfold dilutions)

(iii) Dye solutions

(a) Neutral red; 0.1%

(b) Toluidine blue; 0.1%

(c) Methylene blue; 0.1%

(d) Crystal violet; 0.1%

(e) Janus green B; 0.1%

(f) Light green SF yellowish; 0.1%

(g) Fast green FCF; 0.1%

(h) Phenol red; 0.1%

(i) Uranin (fluorescein disodium salt); 0.1%

(iv) $^{14}C$-labeled metabolites

(a) Mixture of 15 amino acids (U-$^{14}C$) in salmonid saline

(b) D - Glucose (1-$^{14}C$) in salmonid saline

(v) Chemotherapeutic agents

(a) Ronnel; (0,0,-Dimethyl 0,-2,4,5-trichlorophenyl phosphorothioate)

(b) Ruelene; (4-tert-Butyl-2-chlorophenyl methyl methylphosphoramidate)

(c) Phenoxethol; (2-phenoxy ethanol; = 1-Hydroxy-2-phenoxyethane)

The rationale and details of application of each of these permeability tests are briefly outlined as follows:

(i) Ionic solutions:

(a) 0.01 M $AgNO_3$ in distilled water was used as described by Croghan (1958a) to demonstrate permeable areas of crustacean cuticle. $Ag^+$ ions combine at point of contact with $Cl^-$ ions leaking across permeable cuticle from internal body fluids, forming a

deposit of insoluble AgCl in the cuticle matrix. The precipitate is then reduced to a readily visible deposit of black silver, either by exposure to strong light (e.g. a microscope dissecting lamp) or by use of diluted photograhic developer (in this instance a standard darkroom stock solution of D-76 (Kodak) diluted 1:10 with distilled water to prevent osmotic disruption of the specimens). Both methods produced very similar results, though the D-76 technique was preferred. Copepods were washed in distilled water for 15 min. to remove any free $Cl^-$ ions from the body surface and the lumina of the bulla and second maxillary ducts, incubated in the $AgNO_3$ solution for 20 min., then rinsed in distilled water for an additional 15 min. to remove any undeposited $Ag^+$ ions, before developing the AgCl deposits in the D-76 solution for 5-10 min. Incubation in $AgNO_3$ solution and subsequent rinsing were done in darkness to prevent extraneous reduction and deposition by light of silver other than that precipitated by chloride ions. After initial examination with a dissecting microscope, copepods were fixed in formalin (10%), dehydrated in an ethanol series, cleared in xylene and re-examined in either xylene or cedarwood oil. This process enhanced the visibility of the black silver deposits by rendering the copepod transparent.

(b) $KMnO_4$ solution (saturated in distilled water) was used as an independent check on the previous technique, and demonstrates cuticle permeable to $MnO_4^-$ ions by penetration and oxidation of the underlying epidermal tissues, which acquire a "burnt" brown appearance (Croghan, 1958a). No preliminary washings

are needed and impermeable (usually wax-coated) cuticle does not oxidize and darken noticeably with incubation times of up to 15 minutes. Any adhering host mucus and tissue debris not removed prior to treatment will be reaidly oxidized to a dark brown colour, but becomes brittle in the process and is easily detached from the cuticle surface after treatment, thus being clearly distinguished from a true positive reaction with tissues underlying permeable cuticle. As previously, treated specimens were fixed, dehydrated and cleared to increase visibility of the darkened areas.

(c) Salmonid physiological saline is a freshwater teleost saline recommended for salmonids and was made up as described on p. 262 of Hoar and Hickman (1967). This solution was used both as an experimental medium and as a routine incubation bath for holding and maintaining copepods between times of dissection from the host and experimental utilization. Frequently salmonid saline was used as the control treatment in experiments involving other substances. In the permeability studies it was employed as either the tube medium or the external medium of the capillary tube/vial system, and its effect in prolonging activity and survival time of the copepods _in vitro_ was compared with that of distilled water and an isosmotic sucrose solution. The copepods were examined periodically and their condition and approximate times of death were noted, death being defined in _Salmincola_ for these and subsequent experiments as a lack of any visible activity (e.g. maxillary contractions, lateral

swaying and antiperistaltic movements of the gut, movements of the antennae and mouthparts), combined with full extension of the second maxillae and permanent expansion of the contractile spherical portion of the anterior midgut. Specimens displaying only some of these symptoms or occasional spasmodic rather than rhythmic movements of the gut, maxillae, antennae and mandibles were recorded as being moribund.

(d) A stock solution of 0.1 M $MgCl_2$ was made up in distilled water and portions were diluted with salmonid saline to give additional concentrations of 0.05 M and 0.01 M $MgCl_2$. Adult female S. californiensis were exposed to each of these solutions in the capillary tube/vial system. Activity and survival times for the $MgCl_2$- treated copepods were compared with activity and survival times for control animals exposed in the same manner to distilled water and salmonid saline to which equivalent amounts of $Na^+$ (as NaCl) had been added, rather than $Mg^{++}$.

(ii) Metabolic inhibitors:

(a) A stock solution of 0.01 M KCN in salmonid saline was used to prepare further concentrations of 0.001 M, 0.0001 M and 0.00001 M KCN by serial dilutions with additional saline. Each of these solutions was presented to adult female S. californiensis mounted in capillary tubes, as the tube medium of the capillary tube/vial system, and the activity and survival times of treated copepods were compared with activity and survival times of control animals exposed in similar manner to salmonid saline containing equivalent concentrations of KCl rather than KCN.

(iii) Dye solutions:

A number of water-soluble dyes were presented as vital stains to capillary tube-mounted adult female S. californiensis, either via the bulla alone as the medium within the capillary tube or via the external surface excluding the bulla, as the external medium. All dye solutions (0.1% $^{w}/v$) were prepared in either salmonid saline or distilled water, depending upon whether they were to be used as tube media or external media, respectively. Both acidic and basic dyes were used, ranging in (un-ionized) molecular weight from 289 to 809, as indicated below (data from Gurr, 1965).

(a) Neutral red; mol. wt. 289, ionic wt. 253.5, basic (cationic)

(b) Toluidine blue; mol. wt. 306, ionic wt. 270.5, basic (cationic)

(c) Methylene blue; mol. wt. 320, ionic wt. 284.5, basic (cationic)

(d) Crystal violet; mol. wt. 408, ionic. wt. 372.5, basic (cationic)

(e) Janus green B; mol. wt. 483, ionic wt. 447.5, basic (cationic)

(f) Light green SF yellowish; mol. wt. 793, ionic wt. 747 acidic (anionic)

(g) Fast green FCF, mol. wt. 809, ionic wt. 763, acidic (anionic)

(h) Phenol red, mol. wt. 376, ionic wt. 353, acidic (anionic)

(i) Uranin (fluorescein disodium), mol. wt. 376, ionic wt. 330, acidic (anionic)

(iv) $^{14}C$-labeled metabolites

In order to determine if Salmincola could obtain organic molecules from its host via the bulla, adult female S. californiensis were mounted in capillary tubes in replicate groups of ten copepods each and exposed via the bulla only to solutions of $^{14}C$-labeled amino acids or glucose in salmonid saline, by incubation with one of the following tube media.

(a) A mixture of 15 pure U-$^{14}$C-amino acids, combined in concentration ratios simulating a typical algal protein hydrolysate (ICN Pharmaceuticals Inc.). The stock solution was diluted in a ratio of 1 vol: 19 vols. with salmonid saline, from an initial activity of 100 μCi/ml. to a final test solution activity of 5 μCi/ml. Concentrations of individual amino acids in the test solution ranged from 6.1 μM for L-valine (present in largest proportion, i.e. 14.5% of total amino acid content) to 0.16 μM for L-histidine (present in smallest proportion, i.e. 0.5% of amino acid content). Full details of the amino acid mixture and the final test solution concentrations presented to Salmincola are listed in Appendix II.

(b) A solution of 1-$^{14}$C-d-glucose (specific activity 45 mCi/mμ) diluted with salmonid saline in a ratio of 1 vol.:19 vols. from an initial activity of 100 μCi/ml. to a final test solution activity of 5 μCi/ml. The total concentration of glucose (all as 1-$^{14}$C-glucose) in this solution was calculated from the radiochemical analysis data supplied by ICN to be 0.11 mM.

Capillary tubes containing copepods were first filled with salmonid saline, until all members of an experimental group had been mounted, at which time the saline was replaced with the labelled solution. Following incubation at 10$^{\circ}$C for the appropriate time period (Table I) copepods were removed from the vials, rinsed briefly in distilled water, killed and frozen by immersion in liquid nitrogen, and removed cleanly from the capillary tube while still frozen by breaking the manubrium of the bulla, using fine forceps. (This method leaves the bulla and frozen labelled tube medium still contained securely within the capillary tube, thereby simplifying disposal and

preventing any possible contamination of the external surfaces of the copepod with $^{14}C$ label.) Copepods were kept frozen on dry ice, then freeze-dried under vacuum. The second maxillae were removed from dried specimens by dissection, thus eliminating any label contained within the ducts of the maxillae but still actually external to the copepod, and the remainder of the bodies were individually weighed, then stored in a desiccator at -30°C until evaluation by liquid scintillation counting.

Table 1. Exposure times for $^{14}C$-labelled solutions.

(i) $^{14}C$-amino acid uptake (n=20).

| Group 1 | Group 2 | Group 3 | Group 4 | Group 5 |
|---|---|---|---|---|
| 0 hr. (control) | 3 hr. | 6 hr. | 9 hr. | 12 hr. |

(ii) $^{14}C$-glucose uptake (n=20).

| Group 1 | Group 2 | Group 3 | Group 4 |
|---|---|---|---|
| 0 hr. (control) | 4 hr. | 8 hr. | 12 hr. |

(iii) $^{14}C$-amino acid uptake and incorporation into protein (n=10).

| Group 1 | Group 2 | Group 3 |
|---|---|---|
| 0 hr. (control) | 3 hr. | 12 hr. |

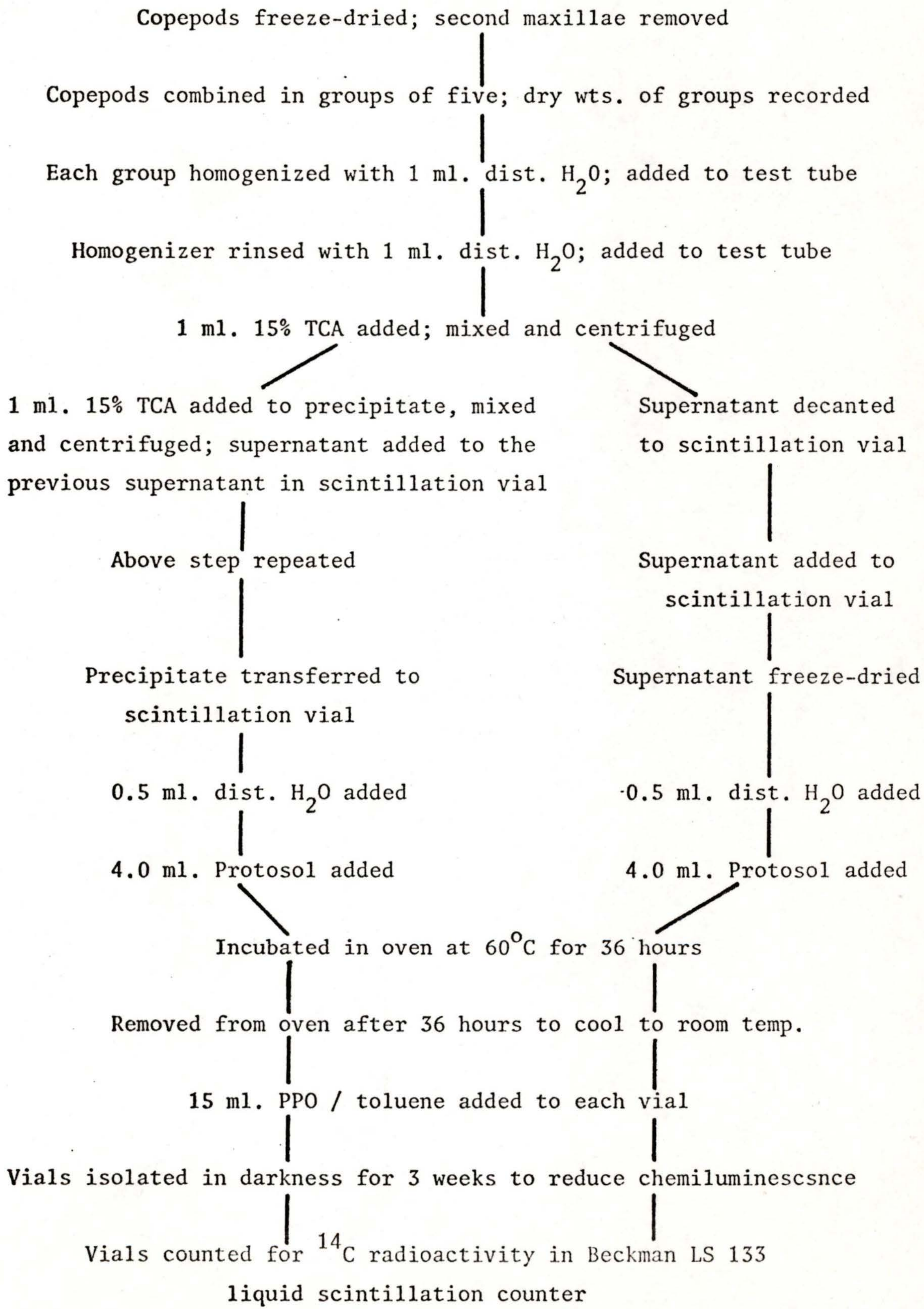
Whole body counts were performed following the methods developed by Chapman (1976) for free-living marine copepods (Calanus plumchrus), using the same liquid scintillation counter (Beckman LS 133), counting efficiency 95$\pm$ 3% as determined by argon-toluene standards, and the same tissue solubilizer (Protosol; New England Nuclear) and fluor (5 grams PPO dissolved

in one litre of toluene). Individual copepods (less the bulla and second maxillae) were solubilized in scintillation vials with 2 ml of Protosol for 36 hrs at 60°C; 15 ml of PPO-toluene was added to each vial after cooling to room temperature, and the vials were then stored in the dark for 21 days to allow chemiluminescence to decrease to negligible values (Chapman, 1976) before counting. Counts repeated after additional storage periods of one and two weeks showed no substantial decrease that would indicate further reductions in chemiluminescence with longer storage times.

In addition, two groups of 10 copepods each were exposed via the bulla to the amino acid mixture as above for periods of 3 and 12 hours, respectively, then analyzed to determine levels of $^{14}C$ in the free amino acid pool and incorporated into protein. Following exposure, each group was split into two sub-groups containing 5 animals, and the copepods were freeze-dried, dissected to remove second maxillae, combined to give one sample for each sub-group and weighed. The four tissue samples (plus two control samples of 5 copepods similarly prepared but without exposure to $^{14}C$-amino acids) were homogenized and processed according to the flow diagram (Fig. 2) to separate protein and free amino acid fractions. These fractions were then solubilized as described for whole body counts and counted to determine relative amounts of $^{14}C$ label in the free amino acid and protein fractions. Counts were converted to counts per minute per milligram dry wt. (c.p.m./mg) using subgroup dry weights and compared with respect to exposure time.

Counts were not corrected for individual quenching rates (as was done in Chapman's study) because only relative quantities taken up were

Fig. 2 Flow-diagram for separation of protein and free amino acids.



of concern in these initial experiments, rather than specific uptake rates; these were sufficiently apparent from the uncorrected counts, which likely underestimate slightly the true uptake of $^{14}C$-labelled organic molecules.

(v) Chemotherapeutic agents

Three compounds were tested *in vitro* as potential control agents for *Salmincola*. These included Ronnel and Ruelene, two systemic organophosphate insecticides used primarily for control of warble-fly larvae in cattle, and phenoxethol, a compound recommended for use in low concentrations (100-200 p.p.m.) as a fungus control agent for aquarium fishes (van Duijn, 1967) and used at slightly higher concentrations (approx. 300 p.p.m.) as an anaesthetic for relaxing fish prior to handling (particularly salmonids). Both the Ronnel and Ruelene formulations tested were manufactured and provided by Dow Chemical of Canada, Ltd., while the phenoxethol (practical grade) was produced by Eastman Kodak.

(a) Ronnel: 0,0-Dimethyl 0,-2,4,5-Trichlorophenyl phosphorothioate ($C_8H_8Cl_3O_3PS$; mol. wt. 321.5; $H_2O$ sol. 40 mg/l at 23$^{o}$C) (Appendix IVa) (Spencer, 1968). Two formulations were obtained and tested:

- Ronnel (98+% pure analytical grade as white crystalline powder). Concentrations tested were 10, 20 and 40 mg/l (= p.p.m.).
- Trolene 20L (drug grade; emulsifiable concentrate containing 40% Ronnel in unidentified mixture of solvents and emulsifying agents). Concentrations tested were 10, 25 and 50 ul/l (= 4, 10 and 20 mg/l pure Ronnel). Both formulations were presented to *Salmincola* in either distilled water or salmonid saline, as appropriate to the intended route of absorption.

(b) Ruelene: 4-tert-butyl-2-chlorophenyl methyl methylphosphoramidate ($C_{12}H_{19}ClNO_3P$; mol. wt. 292.1) (Appendix IVa) (Spencer, 1968). Again, two formulations were tested:

- Ruelene (= Crufomate; 92+% pure analytical grade, as white crystalline solid). Concentrations tested were 5, 10, 25, 50, 100 and 200 mg/l (=p.p.m.).

- Ruelene 25 E (drug grade; emulsifiable concentrate containing 25% Ruelene in unidentified mixture of solvents and emulsifying agents). Concentrations tested were 10, 25 and 50 ul/l (= 2.5, 6.25 and 12.5 mg/l pure Ronnel). Though listed as "practically insoluble in water..." (Spencer, 1968), Ruelene (analytical grade, 92+% pure) was found to be appreciably soluble in distilled water, producing ultraviolet absorption maxima at 198, 215, 269 and 276 nm which yielded linear absorption curves when graphed against concentration. These curves indicated a solubility at room temperature (23$^{\circ}$C) of up to about 200 mg/l) (roughly five times the solubility reported above for Ronnel). This figure is used here only as an approximate saturation value to indicate that the pure compound is significantly and measurably soluble in water and to confirm that test solutions made up at concentrations not greater than 200 mg/l did in fact contain all of the organophosphate in solution. It should be noted that Ruelene absorption maxima for distilled water solutions were not stable but decreased slowly and steadily with time, presumably indicating a gradual breakdown or alteration of the Ruelene molecule in solution (since a precipitate did not form). Since this phenomenon is difficult if not impossible to interpret accurately in terms of residual organophosphate anti-acetylcholinesterase activity without extensive additional

experimentation, the UV spectrophotometry technique was not used to monitor Ruelene concentrations throughout the course of an experiment. Instead, all test solutions were freshly prepared at the start of each experiment, and in experiments lasting longer than 24 hours, all test media were replaced with fresh organophosphate solutions at 24 hr intervals.

(c) Phenoxethol (= 2-phenoxyethanol; $C_6H_5OCH_2CH_2OH$; mol. wt. 122) (Appendix IVa).

Phenoxethol is slightly miscible with water, up to 26.7 gm/l at 23°C (Stecher, 1968), beyond which point an unstable emulsion is formed. Concentrations below this value, ranging from 1 ml/l to 20 ml/l, were prepared in either distilled water or salmonid saline for use as either the external medium or tube medium respectively, in the capillary tube/vial system.

As previously, activity of the copepods was monitored periodically throughout each experiment and approximate times of death were recorded. Formulations of the above compound which were found to be toxic to _Salmincola_ _in_ _vitro_ were subjected to a further series of tests involving presentation to sockeye smolts (approx. 50 gm) infected with _Salmincola_, in an attempt to determine if control methods using these compounds might be feasible (see Section VII: Chemotherapy).

## VII. Chemotherapy

On the basis of results of the _in_ _vitro_ permeability tests with chemotherapeutic agents, Ruelene and phenoxethol were selected for further testing. These were presented to _Salmincola_-infected sockeye smolts (approx. 50 gm) by both external dip and oral dose methods, as well as by intra-

peritoneal injection (Ruelene only). Regardless of method of presentation, each test of a particular combination of formulation and concentration involved ten experimental (treated) and four control fish, treated either individually or in pairs. Externally visible Salmincola were observed during treatment for signs of activity whenever possible, fresh mortalities (dead no longer than 30 min. before examination) were examined and the condition of attached copepods was noted, and survivors of the treatments were sacrificed for examination of the parasites. It should be noted that these preliminary experiments were intended only to determine whether Salmincola-control potential exists with one or more of these chemicals and methods of presentation, and hopefully to indicate the most promising of these procedures for further testing and development.

(i) Dip treatments

For each test with a particular Ruelene concentration ten experimental and four control fish were placed in pairs in approx. 40 l (= 10 gallon) glass aquaria. Fish were placed in the tanks the day before an experiment was to begin and left overnight without further disturbance to reduce and hopefully standardize the effects of handling stress (Wedemeyer, 1972). After 24 hours in flowing dechlorinated water without further disturbance, flow to the tanks was shut off, the tank volume was siphoned down to the 30 l mark, and the required amount of Ruelene (dissolved in 5.0 ml of 95% ethanol) was added and mixed with the tank volume by continued circulation from the airstone. Ruelene concentrations of 1, 2, 5, 10, 15 and 25 mg/l were tested. Fish were observed continually for the first 30 min., then periodically throughout the duration of the experiment. In experiments lasting longer than 24 hours, test solutions were replaced at

24 hr. intervals by first flushing the tanks for 15 min. with flowing water, followed by shut-off and re-establishment of test concentrations as at the beginning of an experiment. This procedure minimized disturbance of the fish and eliminated unnecessary handling-induced stress.

A similar set of experiments was performed using Ruelene 25E emulsifiable concentrate rather than Ruelene/95% EtOH, at concentrations of 5, 10, 20, 50 and 100 μl/l (corresponding to Ruelene concentrations of 1.25, 2.5, 5, 12.5, and 25 mg/l, respectively).

Phenoxethol dip treatments were generally of much shorter duration, and were designed to approximate typical procedures for anaesthetizing fish prior to handling (for which purpose phenoxethol has often been used by fisheries personnel). The concentrations tested (0.2, 0.33, 1.0, 2.0, and 5.0 ml/l) were made up in 30 l tank volumes in glass aquaria, with aeration, and infected fish were added one at a time, observed until completely anaesthetized, then removed and examined. Anaesthetized fish were then allowed to recover in flowing dechlorinated water for 15 min., at which time mortalities and half the survivors were removed and their parasites examined. Remaining survivors were removed and examined after 48 hours. Control fish (n=4) for each experiment were transferred to a static tank volume of 30 l, with aeration, left for the appropriate period of time, then transferred back to flowing water, to duplicate any effects of handling.

It should be noted that $LC_{50}$'s inferred by results of these preliminary experiments are only very rough approximations based on small sample size (n=10) and crowded experimental conditions, and are therefore not strictly comparable to statistically valid $LC_{50}$'s as determined by standard methods (Sprague, 1969). The volume requirements of these methods (one

litre of test solution per gram of fish tissue per 24 hr. period) could not be met with the available facilities and amounts of test compounds, using 50 gm sockeye smolts.

(ii) Oral dosing

Ruelene, Ruelene 25E and phenoxethol were given orally to infected sockeye smolts in a first attempt at systemic control, using pink gelatin capsules loaded with the test compound and a filling of moist-pellet fish food as the vehicle for the dose. In most experiments these were ingested voluntarily by infected fish trained to accept capsules containing food pellets, thereby eliminating handling problems, though one series of tests combined anaesthesia by dip treatment in 0.33 ml/l phenoxethol and force-feeding with phenoxethol-containing capsules. Regardless of feeding method, acceptance of the oral dose was confirmed by appearance of pink dye from the capsule in the faeces within 24 hrs., while rejection was indicated by either direct observation of regurgitation or appearance of intact portions of the capsule in the tank. Control groups were unnecessary with voluntary acceptance since each fish serves as its own control during the training period, but controls receiving capsules containing food pellets only were used in force-feeding experiments to eliminate any possible effects of handling.

Fish were contained singly in 20 l tank volumes of flowing dechlorinated fresh water and given three identical doses at 24 hr. intervals. Following final treatment, fish were left undisturbed for 48 hrs., then sacrificed to determine the condition of the attached Salmincola. The doses of each compound administered are indicated in Table II.

Table II. Oral doses of Ruelene, Ruelene 25E and phenoxethol administered to sockeye smolts infected with Salmincola. (Three doses per fish at 24-hour intervals.)

| Chemotherapeutic Compound | Volume of Dose | Weight of Pure Compound | Dose per kg. body weight per 24 hrs. |
|---|---|---|---|
| Ruelene | - | 1.0 mg | 20 mg |
| | - | 2.5 | 50 |
| | - | 5.0 | 100 |
| | - | 10.0 | 200 |
| Ruelene 25E | 10 μl | 2.5 mg | 50 mg |
| | 20 | 5.0 | 100 |
| | 50 | 12.5 | 250 |
| Phenoxethol | 10 μl | - | 0.2 ml |
| | 25 | - | 0.5 |
| | 50 | - | 1.0 |
| | 75 | - | 1.5 |
| | 100 | - | 2.0 |

(iii) Intraperitoneal injection (Ruelene only)

Pure Ruelene was given by intraperitoneal injection at four dose levels to infected sockeye smolts in an attempt to by-pass possible breakdown and deactivation in the intestine after oral dosing. Prior to inoculation, fish were anaesthetized in 0.33 ml/l phenoxethol solution, and the needle was inserted into the body cavity ventrally, just anterior to the pelvic fins. Fish were injected three times at 24 hr intervals with either 0.25, 0.5, 1.0, or 2.0 ml of a 200 mg/l stock solution of pure Ruelene in salmonid saline.

Hence fish received three identical doses of 50, 100, 200 or 400 μg Ruelene per injection, respectively, corresponding to dose levels of approx. 1, 2, 4, and 8 mg/kg body wt/24 hr. period for 72 hours. Ten fish were treated at each dose level as in previous tests (four control fish receiving equivalent volumes of salmonid saline without Ruelene), and were sacrificed for examination of the attached copepods 48 hours after the final injection.

# RESULTS

## I. General Structure and Organization of the Bulla and Associated Ducts.

The bulla or "attachment organ" of Salmincola is a mushroom-shaped structure consisting of an almost discoid anchor approximately 1.5-2 mm. in diameter, permanently embedded in host tissues from the time of attachment, and a short manubrium or "stalk" extending just above the surface of these tissues, to which the adult female copepod is cemented by the tips of the second maxillae (Fig. 3 a,b,c,; Plate Ia). It becomes the functional attachment structure of the parasite at the time of the final moult into the adult female, and is a key identifying feature of almost all adult female Lernaeopodidae. The attachment process and the external structure of the bulla and second maxillae have been described previously (Kabata and Cousens, 1973).

Within the bulla two narrow passages extend downwards from small openings in the tip of the manubrium and enter a large horse-shoe shaped space located within the discoid portion of the anchor, close to the subanchoral surface (Fig. 3c). The "loop-duct" thus formed connects two large cuticle-lined ducts, one of which extends up the centre of each second maxilla (Fig. 3 a,b,). These second maxillary ducts communicate with the loop-duct via hollow plugs at the tips of the maxillae, which are cemented securely into the two openings in the manubrium during the attachment process, thus sealing the system (Fig. 3a). Near the base of each second maxilla the proximal end of the maxillary duct expands to form a contractile cuticle-lined sac. From this sac a small cuticular funnel leads into a narrow cuticle-lined tube, here termed the proximal maxillary duct, which passes through the base of the maxilla and continues into the trunk region of the copepod, terminating in a series of small lobular glands

Fig. 3 Diagrammatic representation of the bulla and second maxillary duct system of *S. californiensis*.

a) Anterior view of bulla and duct system.

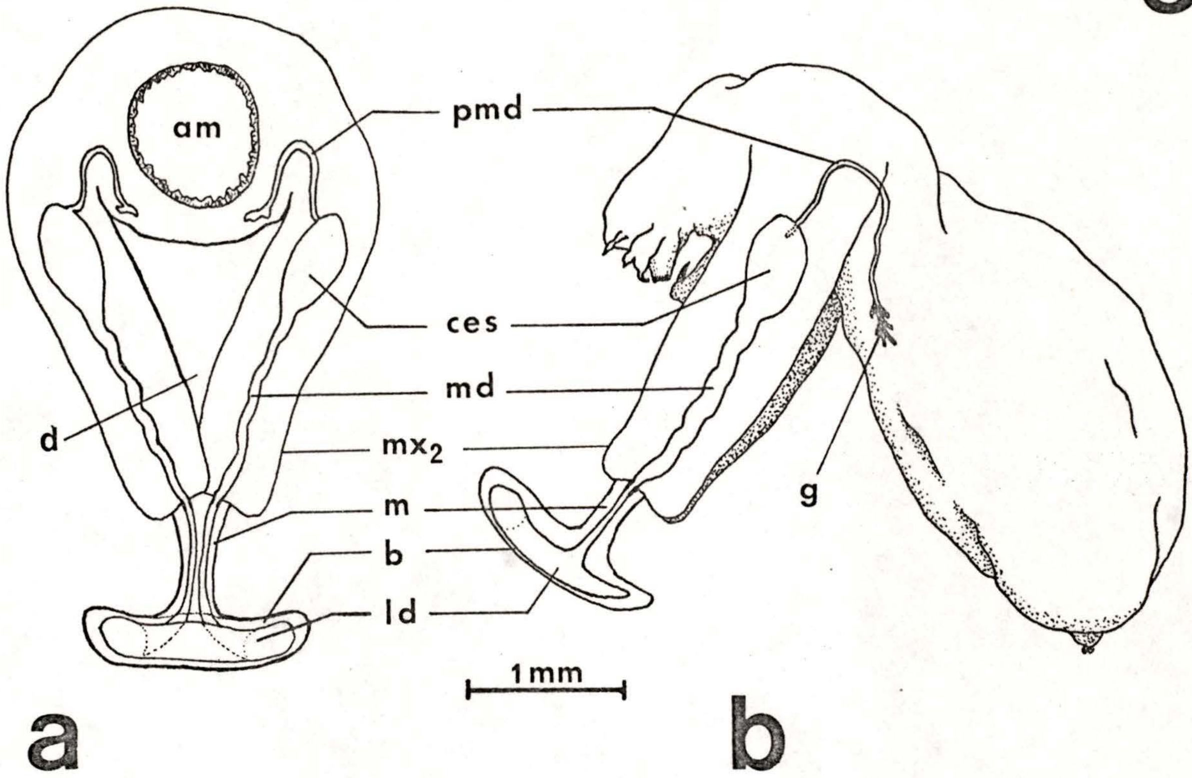
b) Lateral view of bulla and duct system.

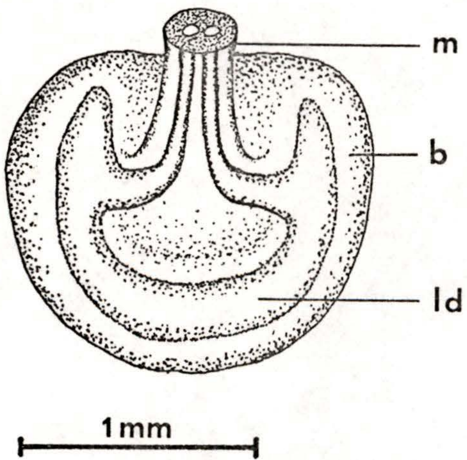
c) Bulla (manubrium cut at base).

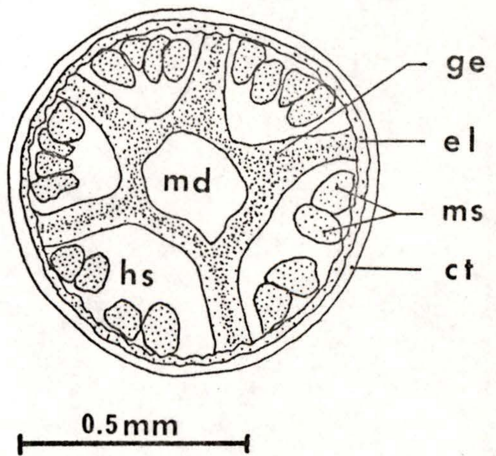
d) Section across second maxilla.

am - anterior midgut; b - bulla; ces - contractile end sac of second maxillary duct; ct - external cuticle; d - location of section shown in (d); el - epidermal layer; g - terminal glands of proximal maxillary duct; ge - glandular epithelium of maxillary duct; hs - haemal spaces of second maxilla; ld - loop duct of bulla; m - manubrium of bulla; md - second maxillary duct; ms - longitudinal muscle bands of second maxilla; $mx_2$ - second maxilla; pmd - proximal maxillary duct.

3




c

d

situated along the lateral walls of the haemocoel (Fig. 3 a,b).

(i) The Bulla

The bulla is formed as the secretion product of a gland in the frontal region of the chalimus IV female larva, and after expulsion at the final moult, implantation, reattachment to the tips of the second maxillae, and inflation to its final proportions, it exists as a toally acellular non-living structure embedded within the surface tissues of the host fish. It often acquires a thin reticular coating of host connective tissue fibers, but only rarely generates any further host tissue response such as encapsulation (Kabata and Cousens, 1977), and usually appears to be accepted by host tissue as "self" rather than as an irritating foreign body.

The bulla appears firm, elastic or rubber-like, colourless and semi-transparent in both live and formalin-preserved specimens. It fractures and tears readily along its longitudinal axis, but is otherwise tough, resilient and tolerant of considerable handling during experimental maniuplations (e.g. insertion into a capillary tube tip). It is most resistant to stresses applied parallel to the long axis of the manubrium, and only slightly less resistant to shearing or twisting forces, such that a firm pull directed along the long axis at the manubrium often causes the bulla to be drawn from its cavity within the host tissues without breaking. It is thus well adapted to withstand the twisting movements of the parasite and the pull of a passing stream of water on the trailing body of the copepod, the major forces to which it would likely be exposed in nature. The manubrium is particularly tough and dense, usually appearing a yellow or amber colour by transmitted light and brown by

reflected light. This appears to be due to some form of chemical hardening, perhaps wholly or in part the result of penetration of the matrix of the manubrium by the cement with which it is firmly attached to the hollow plugs at the tips of the second maxillae.

The observations of both strength and ability to fracture and tear along the longitudinal axis suggest a fibrous nature with fibers running parallel to the long axis, and this is confirmed by light microscope studies. Paraffin sections stained with either Mallory's triple-stain or haematoxylin and eosin reveal the structure to be composed of densely packed parallel fibers forming a reticulum in the discoid anchor portion (Plate Ia, b). The fibrous matrix stained blue with aniline blue of Mallory's and pink with eosin, the more dense manubrium staining more darkly in both cases. Both techniques also revealed a thin outer covering of dense darker staining, apparently homogenous material previously termed the pellicle (Kabata and Cousens, 1972), but a layer of material lining the loop duct could not be demonstrated in any part of the bulla (Plate Ib).

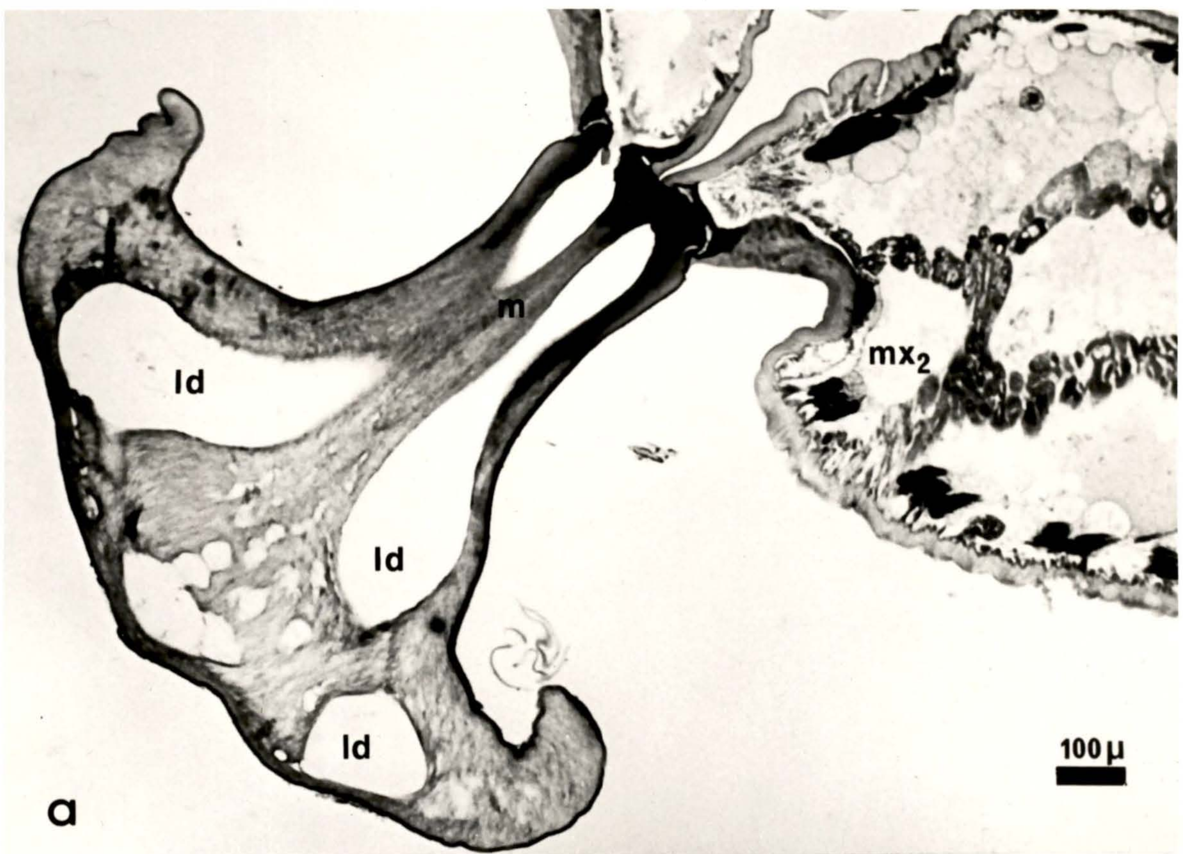
A series of histochemical tests carried out on paraffin sections of the bulla indicates that it is composed of a mixture of neutral and acid mucopolysaccharides and mucoproteins (Table III). The positive periodic acid-Schiff and Mayer's mucicarmine reactions are indicative of neutral mucopolysaccharides and mucoproteins, while the positive response with alcian blue is indicative of acid mucopolysaccharides and sialic acid-containing mucoproteins (Drury and Wallington, 1967). Partial restoration of staining with alcian blue by saponification, following blocking of the reaction by methylation, indicates the presence

Plate I The attachment organ of S. californiensis as seen with the light microscope.

a) Bulla and manubrium attached to tips of the second maxillae (Mallory's one-step triple stain). X 90.

b) Enlargement of part of bulla shown in a), to reveal general structure of fibrous matrix, loop duct and pellicle (Mallory's one-step triple stain). X 300.

b - fibrous matrix of bulla; ld - loop duct; m - manubrium; $mx_2$ - second maxilla; p - pellicle forming outer surface of bulla.


m
ld
mx$_2$
ld
ld
100 μ
a

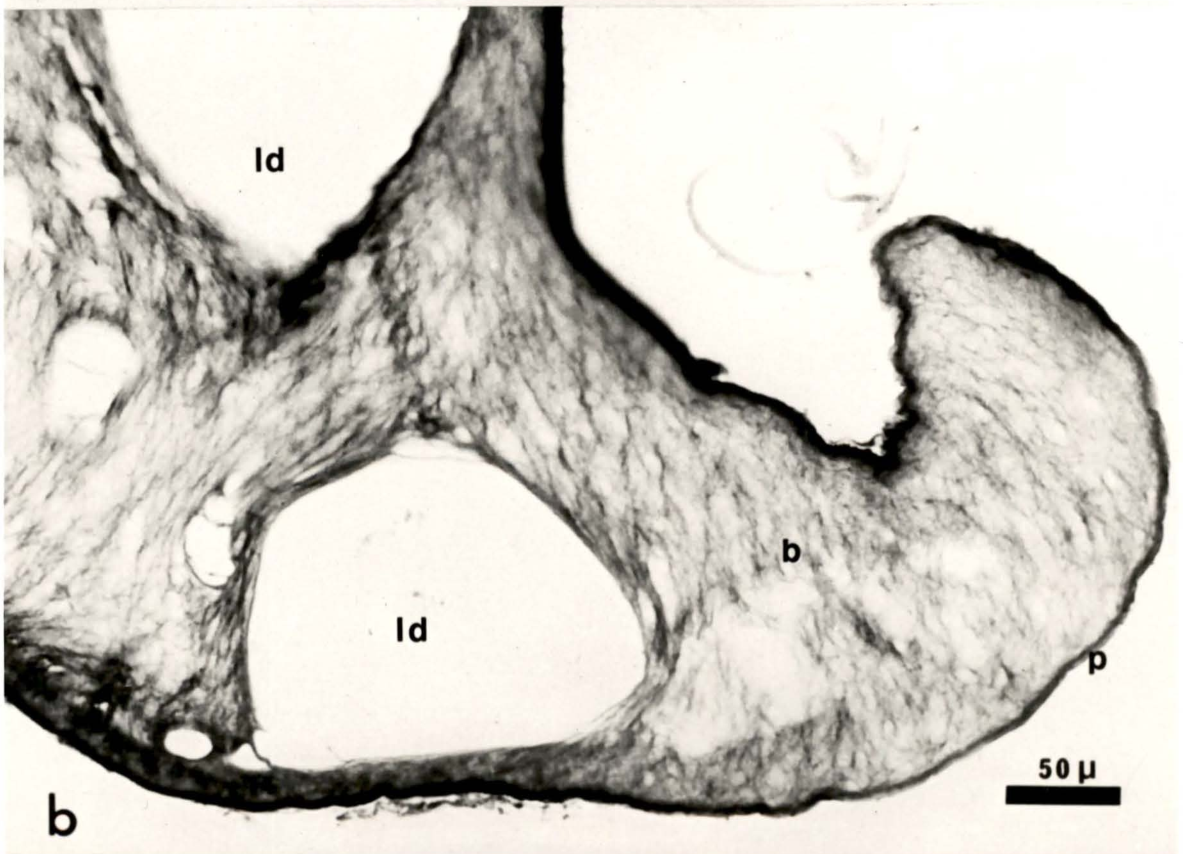

ld
b
ld
p
50 μ
b

Table III. Summary of results of histochemical analysis of the bulla.

(-, negative; +, weakly positive, ++, positive; +++, strongly positive, ±, inconclusive)

| Test | Result | Interpretation |
|---|---|---|
| **I Carbohydrates:** | | |
| (i) P.A.S. test | ++ | |
| control (without periodic acid) | - | no pre-existing aldehyde groups |
| diastase control for glycogen | ++ | no glycogen or starch |
| (ii) Mayer's mucicarmine | ++ | mucins present |
| (iii) Alcian blue | + | acid mucopolysaccharides and/or |
| after methylation | - | sialic acid mucoproteins |
| after methylation and saponification | + | some simple acid mucopolysaccharides |
| (iv) Uranyl nitrate - Azur A methachromasia | ± | inconclusive; acid mucopolysaccharides not abundant |
| **II Proteins** | | |
| (i) Millons reaction (for protein tyrosine) | + | some tryosine-containing protein present |
| (ii) Mercuric bromophenol blue | + | some protein present |
| (iii) Ninhydrin test | ± | inconclusive |
| **III Specific Tests** | | |
| (i) Chitin test | - | little or no chitin present |
| (ii) Alizarin Red S (for $Ca^{++}$) | + | some bound $Ca^{++}$ present |

of some simple acid mucopolysaccharides. The remainder of the initial positive alcian blue response presumably is due to either complex acid mucopolysaccharides or sialic acid-containing mucoproteins, neither of which show restored alcian blue staining after methylation and subsequent saponification (Drury and Wallington, 1967). Argument in favour of the mucoproteins is suggested by the lack of detectable metachromasia (due to acid mucopolysaccharides) with Toluidine blue and uranyl nitrate-azur B, implying that acid mucopolysaccharides are not major components of the structure (though apparently present in small amount, as noted above). This argument is further supported by the positive reactions with Millon's reagent (specific for tyrosine) and mercuric bromophenol blue, both of which demonstrate protein (Humason, 1967), though the less sensitive ninhydrin test was inconclusive.

A specific test for chitin (Gurr, 1965), a neutral mucopolysaccharide-calcium complex commonly found in crustacea, failed to demonstrate its presence in the bulla, though a weak positive reaction with alizarine red S indicated the presence of small amounts of bound calcium (Humason, 1967).

Both scanning and transmission electron microscope studies of the bulla supported earlier suggestions of the fibrous reticular nature of its structure. Scanning E.M. examination of the cut face of a portion of the disk of the bulla revealed a highly porous matrix of fine tightly interwoven filaments. This structure was most apparent and easily demonstrated without preparation artifacts at the inner surface of the bulla loop-duct, which closely resembles the surface of a fibrous sponge (Plate II,a). The duct is not lined in any manner and merely represents a space within the fibrous matrix of the bulla, the lumen of which is continuous with

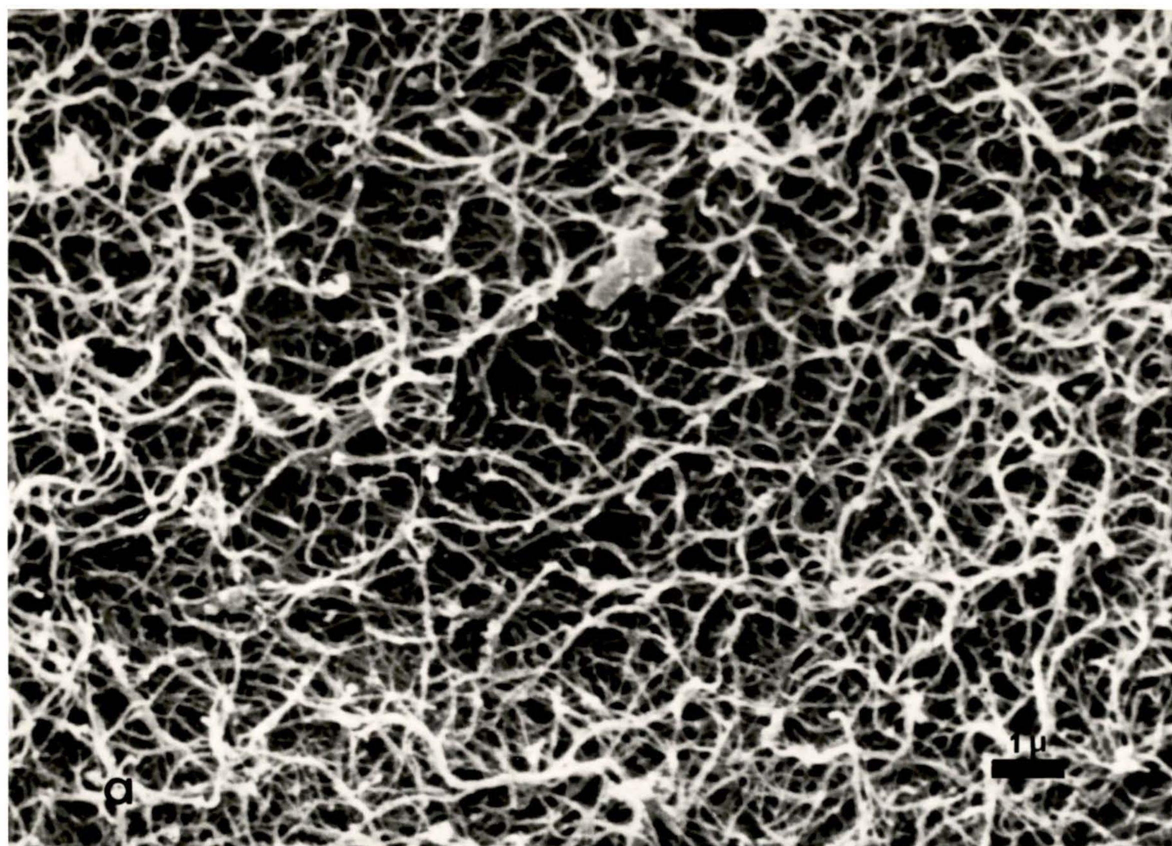
Plate II    Ultrastructure of the bulla loop-duct.

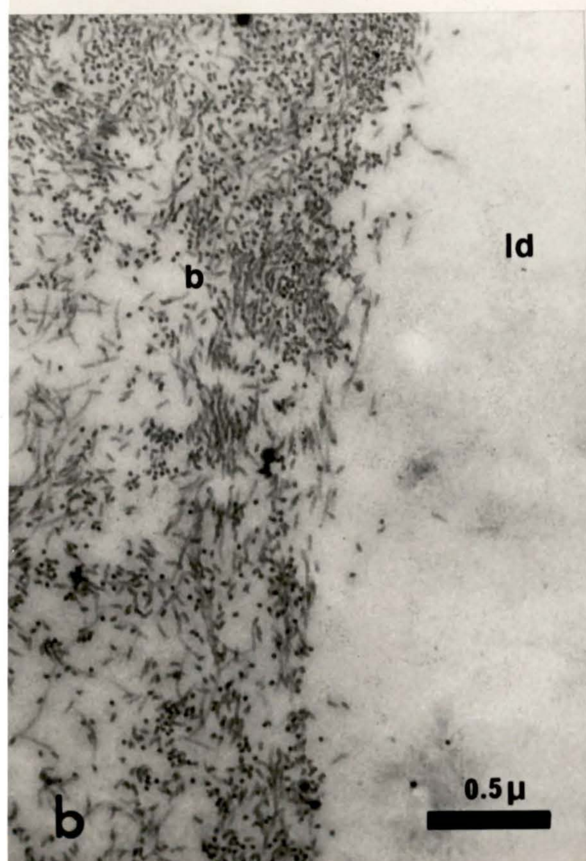
a) Scanning electron micrograph of the inner surface of the loop-duct, revealing the fibrous sponge-like composition of the bulla matrix. (Gold-palladium coated). X 10,000.

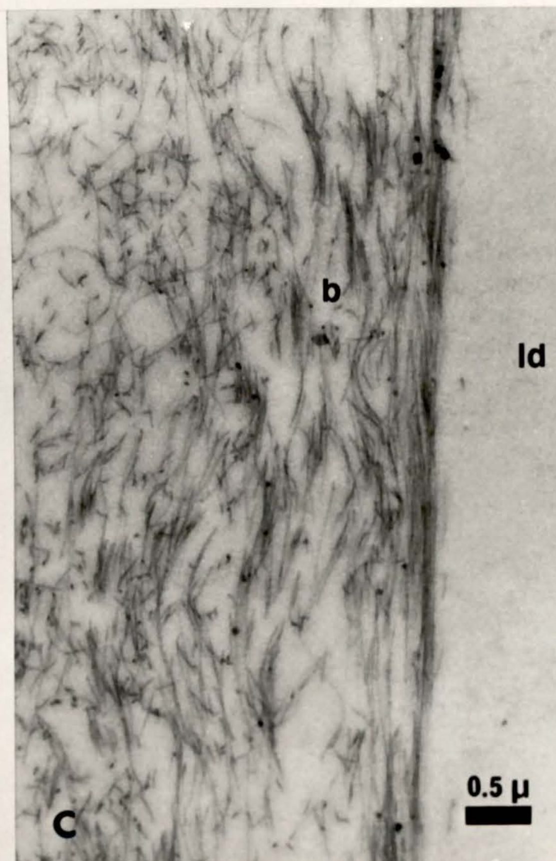
b) Transmission electron micrograph of thin epon section cut perpendicular to wall of loop-duct, demonstrating fibrous nature of matrix and lack of duct-lining or other major structural modification at the interface. X 31,000.

c) Similar to b), but showing parallel orientation of filaments of matrix which often occurs near loop-duct. X 18,000.

b - fibrous matrix of bulla; ld - loop-duct lumen.


1 μ
a


ld
b
0.5 μ
b


b
ld
0.5 μ
c

the spaces between the fibers comprising the matrix. The absence of a lining within the loop-duct was further confirmed by thin epon sections cut perpendicular to this surface and examined with the transmission electron microscope (Plate II, b,c). These indicated a structure composed entirely of fine fibers, which ends abruptly and without modification at the interface with the duct lumen.

The density of the fibrous matrix varies considerably with location within the bulla, being least dense in the central portion of the bulla (Plate III, a), more dense nearer the outer surface (Plate III,b) and most dense immediately below the pellicle bounding the outer surface (Plate IV,a). A slight increase in density also occurs in that portion of the matrix closely surrounding the loop-duct, together with a tendency for most of the fibers to lie parallel to each other (Plate II, b,c).

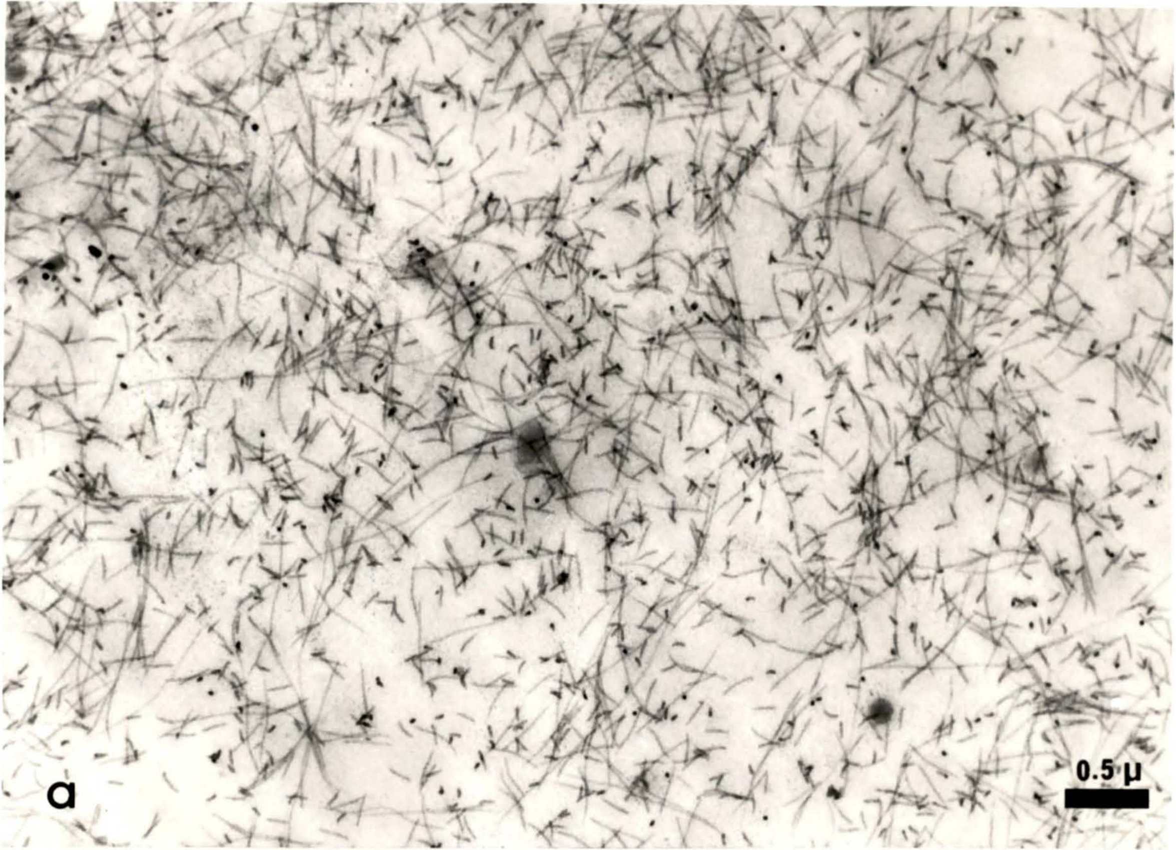
The pellicle, which appears in the light microscope only as a thin layer of dense-staining material bounding the outer surface of the bulla, is seen to have a far more complex and most interesting structure when examined by transmission electron microscopy. The pellicle is composed of a dense-staining homogenous inner layer 0.15-0.2 microns in thickness, and an outer layer or "striated border" of about the same thickness consisting of short parallel fibers arranged perpendicular to the dense inner layer (Plate IV,a). These fibers are closely packed in an irregular pattern, with occasional indications of localized hexagonal packing, as revealed by tangential sections of the pellicle (Plate IV b). In addition, larger fibers are interposed between the short fibers at irregular intervals, arising from the dense inner layer of the pellicle and extending outwards for some considerable distance beyond the striated

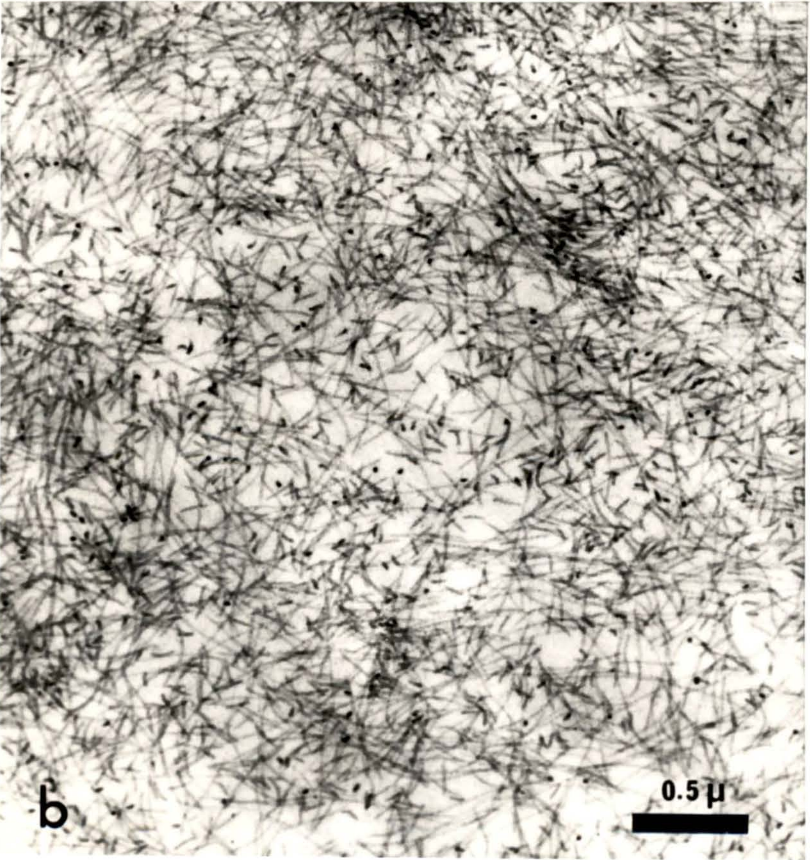
Plate III The fibrous matrix of the bulla.

a) Electron micrograph from central region of bulla matrix. Filaments are loosely interwoven to form an open meshwork. X 22,000.

b) Electron micrograph from peripheral region of bulla. Density of matrix is considerably greater than in a), and increases further near the pellicle at the outer surface (see Plate IV). X 30,000.

c) Higher magnification in same area of bulla as b). The diameter of the filaments appears to be uniform throughout the bulla at approximately 100 Angstroms (0.01 μl X 45,000).


0.5 µ
a

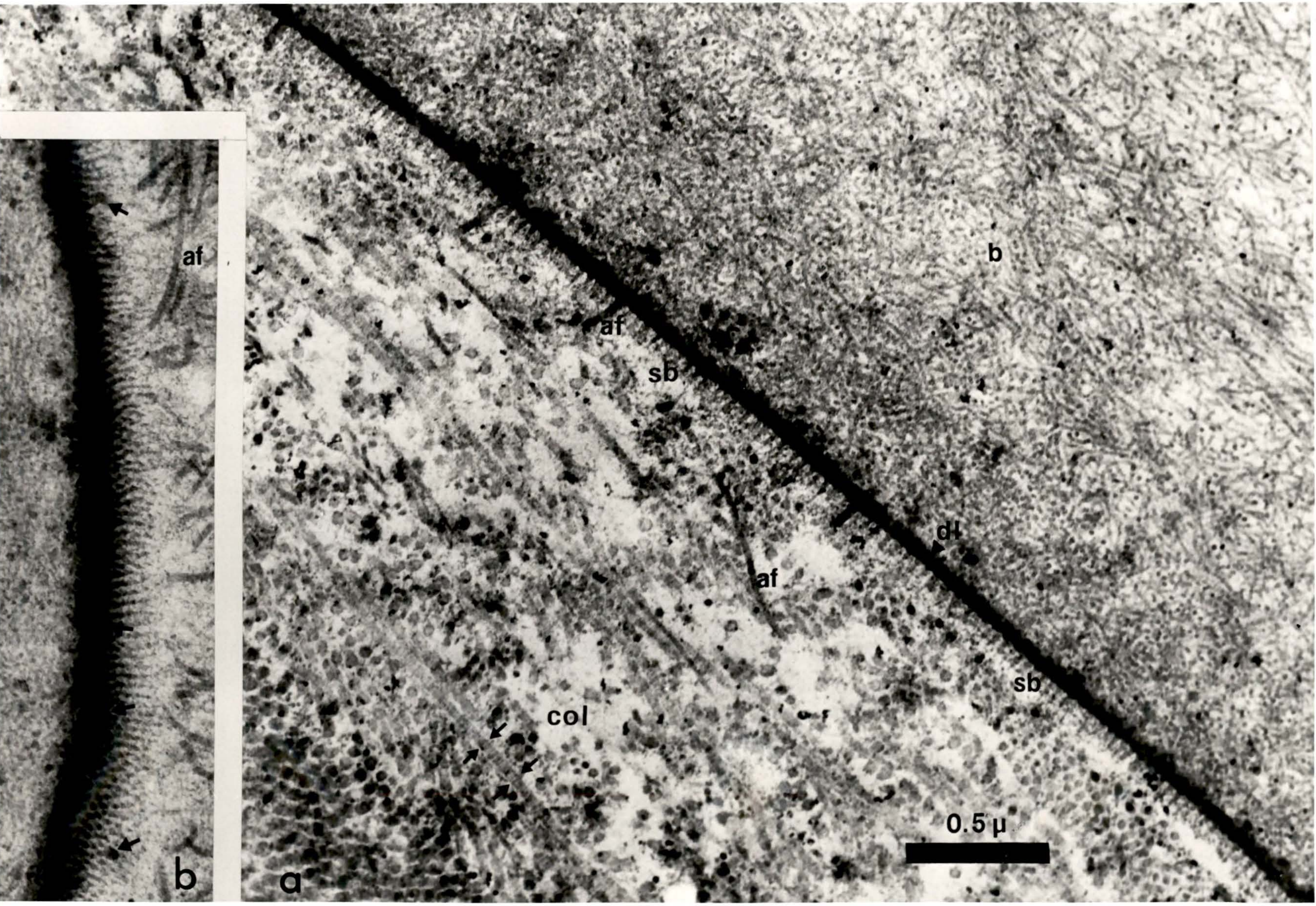

0.5 µ
b

0.5 µ
c

Plate IV Ultrastructure of the pellicle of the bulla: the host-parasite interface.

a) Electron micrograph of a thin section through the pellicle. The bulla matrix is most dense just beneath this outer boundary and appears to fuse with its dense-staining inner layer (dl). This layer is between 0.15 μ and 0.2 μ in thickness, and gives rise to numerous short fibres standing perpendicular to its outer surface which together form a striated border (sb) of a similar thickness. Long, dense-staining "anchoring filaments" (af) of about 300 Angstroms (0.03 μ) diameter also arise from the dense inner layer, extending out between and beyond the short fibres of the striated border to intertwine with the adjacent host collagen fibres (col) which form a thin connective tissue sheath surrounding the bulla. Arrows indicate a collagen fibre showing the characteristic banding pattern. X 48,000.

b) An oblique section through the pellicle reveals the pattern of spacing of the short fibres forming the striated border, as well as the positioning among them of several anchoring filaments (arrows). X 48,000.


af
b
b
af
sb
dl
af
sb
col
0.5 μ
a

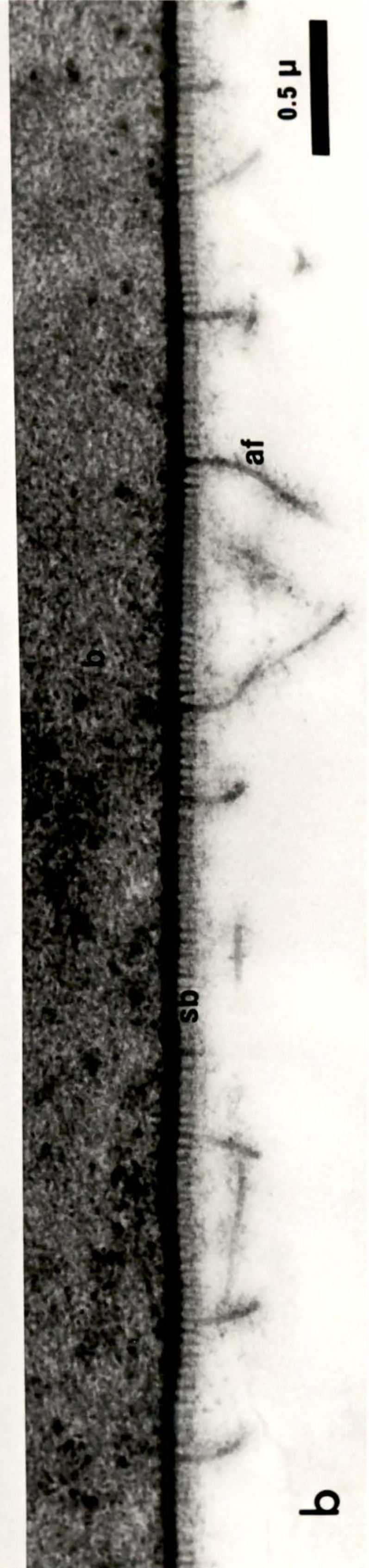
border, to intertwine with the network of collagen fibers laid down by the host over the surface of the bulla (Plates IVa, V). This collagen production presumably occurs as part of the wound-healing process following implantation and results in formation of a thin sheath of connective tissue immediately surrounding the implanted portion of the bulla. It is suggested that the fibrous extensions of the pellicle, by intertwining with the collagen fibers of this thin connective tissue layer, firmly anchor and immobilize the bulla within host tissue without the use of a cement-like substance (as is found in many marine lernaeopodids), thereby preventing movement of the bulla within host tissues which might cause further irritation and result in a more vigorous connective tissue response such as encapsulation. Both infiltration of the bulla and surrounding tissues with a cement substance and encapsulation in a heavy fibrous and possibly calcified sheath would likely prove detrimental to any exchanges of metabolites which may occur across the surface of the bulla.

There is no evidence to support the alternate hypothesis of a host rather than parasite origin for these "anchoring filaments", and the opposing evidence is fourfold: the filaments appear to arise directly from the dense inner layer of the pellicle (Plates IV,V); they are far more dense-staining than the host-produced collagen fibers and lack the characteristic periodicity of banding or striations that on occasion can be observed in the latter (Plate IVa); they decrease in abundance in the collagen layer with increasing distance from the surface of the bulla; and they remain attached to the pellicle and extend outwards from it when the thin adhering layer of collagen fibers is stripped away (Plate Vb).

Plate V Anchoring of the bulla in host tissue.

a) Electron micrograph of section through pellicle and adjacent host tissue showing a number of anchoring filaments (af) intertwining with collagen fibres (col) laid down by the host. Occurring over the entire surface of the implanted portion of the bulla, large numbers of these filaments serve to anchor the bulla firmly into surrounding tissues. X 38,000.

b) As above, but with the surrounding sheath of collagen fibres stripped away completely. The anchoring filaments or at least their basal portions remain attached to the pellicle and project outwards from its surface, indicating the relative strengths of their attachment to the pellicle and host tissues. The filaments and striated border (sb) also appear to possess or to have acquired a coating of less-dense-staining particulate material which might be involved in adhesion to host tissues, the structure of the striated border being particularly approximate for this purpose. X 38,000.


0.5 µ
af
b
sb
b

(ii) The Second Maxillae and Maxillary Ducts

The second maxillae of adult female Salmincola californiensis are elongate cylindrical "arm-like" structures (Fig. 3 a,b) which protrude ventrally from the anterior portion of the trunk, their tips being joined by the manubrium of the bulla. These appendages are apparently unsegmented, highly flexible, contractile along their long axis by means of longitudinal muscle bands, and capable of independent contraction and flexion, thus permitting orientation of the copepod so as to place the mouth against any adjacent host tissue within reach for feeding. Extending up the centre of each second maxilla from the tip almost to the base is a tubular cuticle-lined duct which terminates in a contractile saccular enlargement of the lumen. These ducts are continuous with and joined by the loop-duct through the bulla via the hollow plugs at the tips of the maxillae,thus forming a single long tubular compartment with the loop-duct at its centre (Fig. 3a). This compartment, though partially surrounded by parasite tissue, is bounded by a thin cuticular lining within the second maxillae and thus lies functionally external to the copepod. The remaining central portion of this compartment (distal to the tips of the maxillae) is bounded within the unlined loop-duct by the fibrous matrix of the bulla, beyond which lie the tissues and tissue-fluids of the host fish.

Immediately adjacent to the cuticular lining, the maxillary ducts are surrounded by a layer of modified epithelial tissue, which appears glandular in light microscope studies of paraffin sections stained with either Mallory's triple-stain or haematoxylin-eosin. This tissue also extends outwards from the ducts along both sides of the five supporting

membranes which connect the ducts to the outer walls of the maxillae for their full length, thus appearing penta-radiate in cross section (Fig. 3d; Plate VIa). (The pairs of muscle bands responsible for contraction and bending of the maxillae extend longitudinally through the haemocoelomic spaces between these five rays of tissue, often running a slightly spiral course along the length of the appendage.)

The cells of the "glandular" tissue show well-defined nuclei with both stains used, and often appear multi-nucleate with the light microscope. Individual cell membranes are rarely visible and cell boundaries are generally impossible to determine, giving the entire tissue the appearance of a syncytium, though subsequent transmission E.M. studies indicate this is not the case. The glandular tissue is least developed at the distal end of the second maxilla and becomes more extensively developed towards the base, the greatest development occurring immediately distal to the contractile end sac. At this point the five rays of glandular tissue appear to be composed of a number of closely spaced lobes, separated by narrow channels and often containing many large inclusions which suggest synthesis and perhaps temporary storage of lipid or some other metabolic intermediate (Plate VIb).

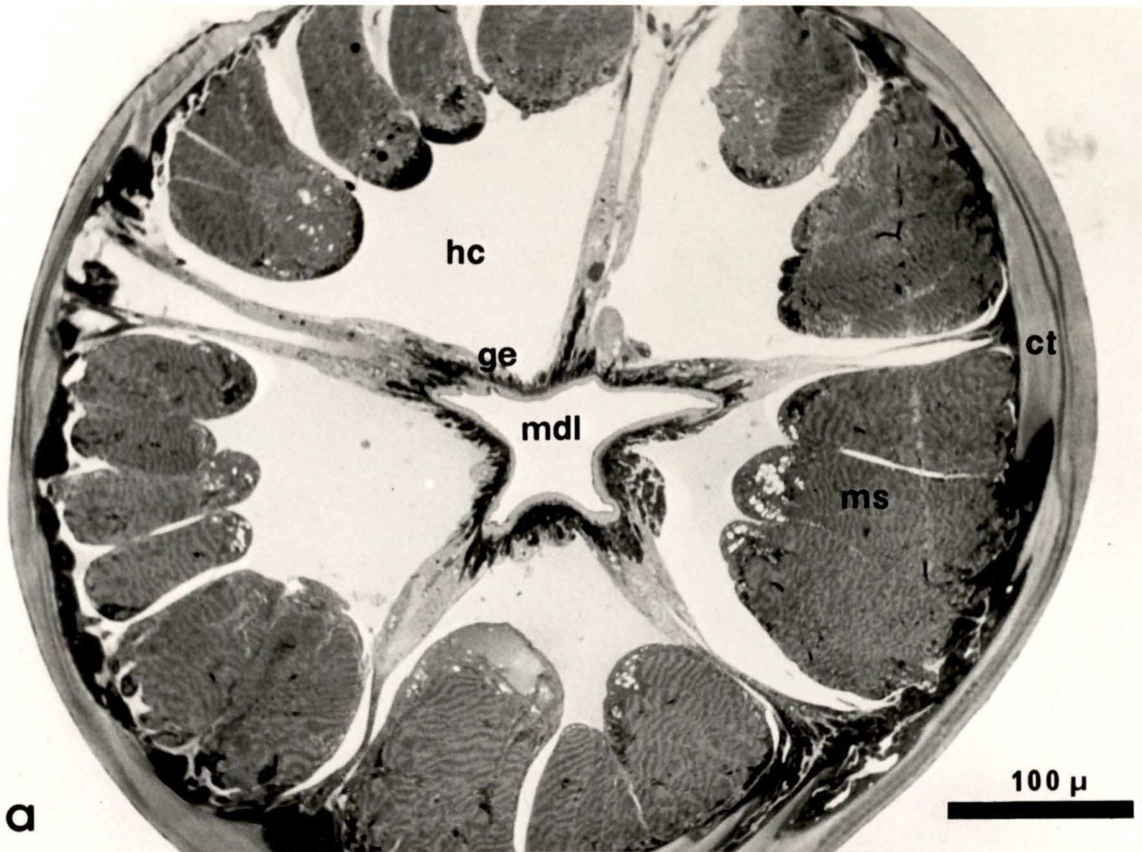
Scanning E.M. study of entire second maxillae, which had been sliced longitudinally after fixation to expose the lumen of the duct, showed the cuticlar lining to be smooth and almost featureless with only slight irregularities along the adlumenal surface (Plate VIIa), except for folds and ridges observed along partially contracted portions of the duct (Plate VIIb). At the cut edge of the duct lining many spaces were observed between the cuticle and the underlying tissue, these often being traversed

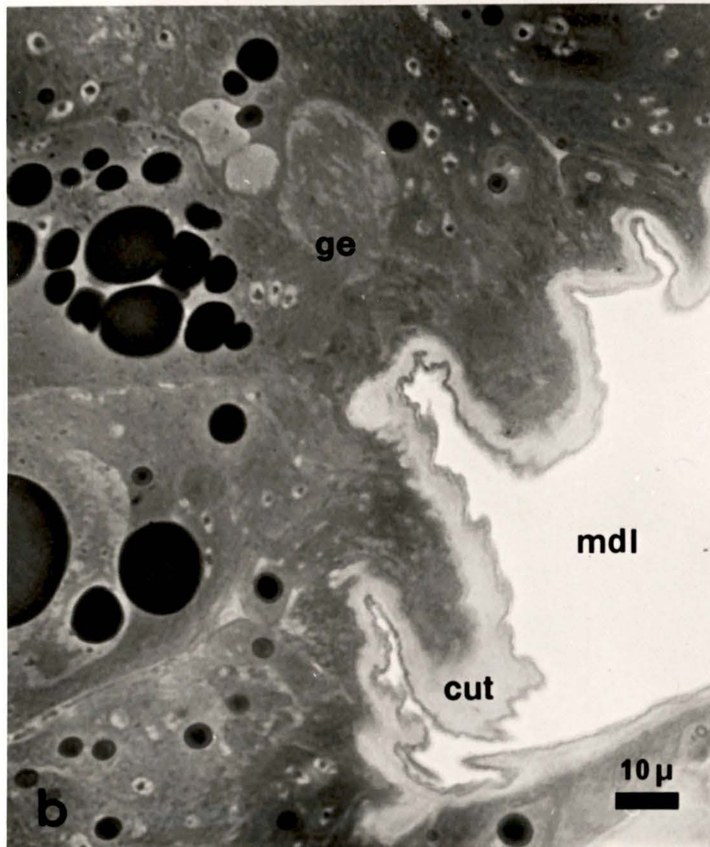
Plate VI General structure of the second maxillae and maxillary duct.

a) Photomicrograph of epon thick section of second maxilla cut in cross-section as indicated diagrammatically in Fig. 3d, illustrating the typical five-rayed arrangement of the glandular epithelial tissue (ge) about the central maxillary duct. Note also the arrangement of longitudinal muscle bands (ms) along the outer walls of the haemocoelomic spaces (hc). (Richardson's stain). X 240.

b) Photomicrograph of glandular epithelium (ge) surrounding maxillary duct (mdl), illustrating the cuticular lining of the duct (cut), a number of lipid droplets within the epithelial cells, and the prominent nuclei of the latter. (Richardson's stain). X 800.

c) As above, further demonstrating the extent of lipid droplet accumulation within cells of the glandular epithelium. (Richardson's stain). X 530.


hc
ge
mdl
ct
ms
100 µ
a


ge
mdl
cut
10 µ
b

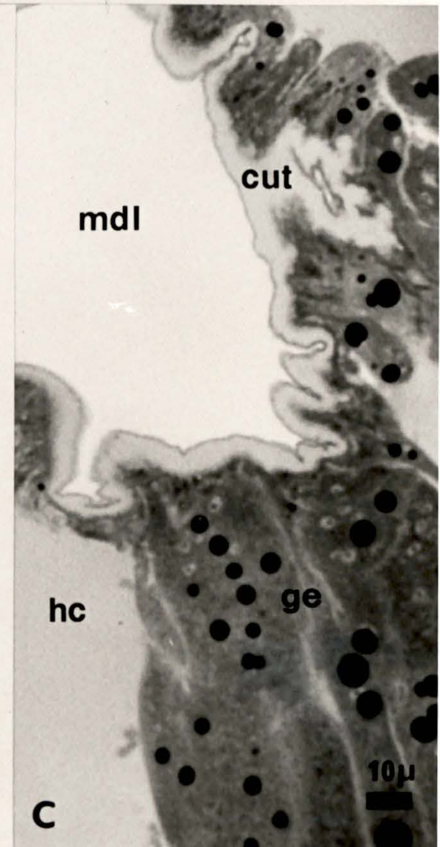
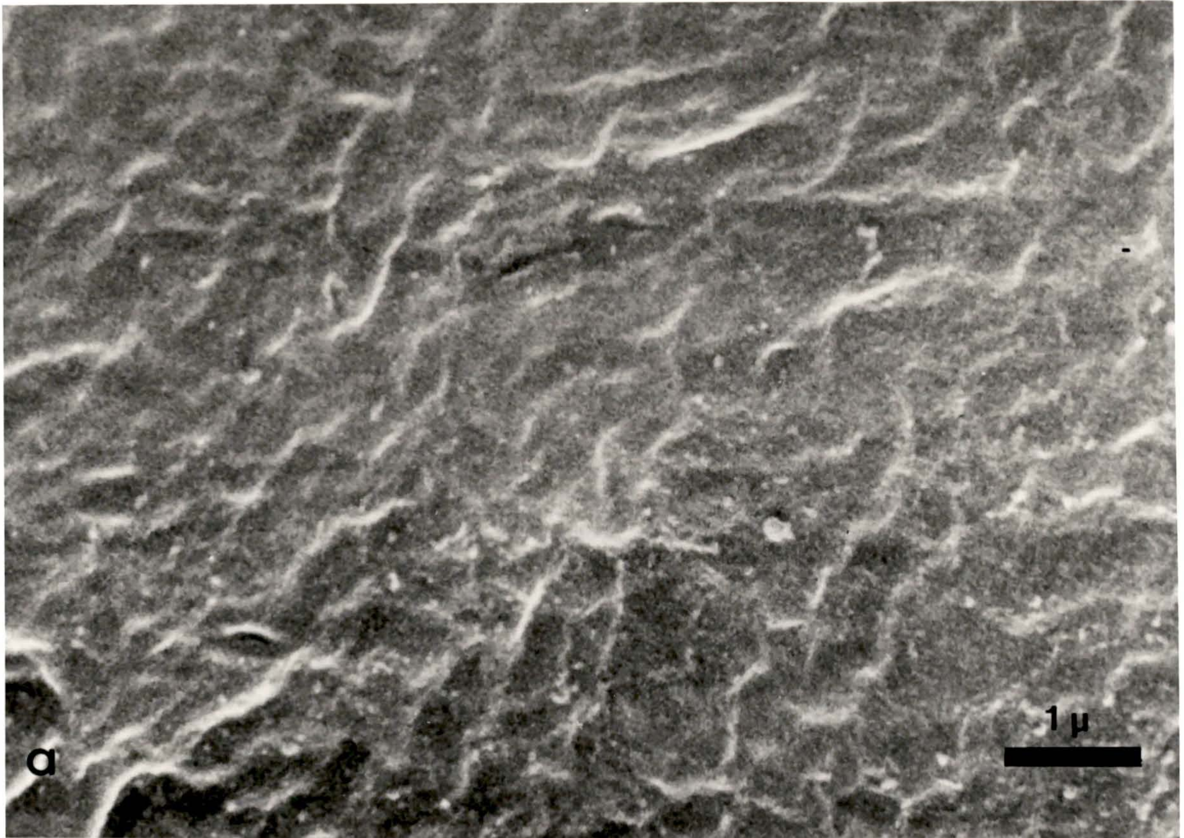
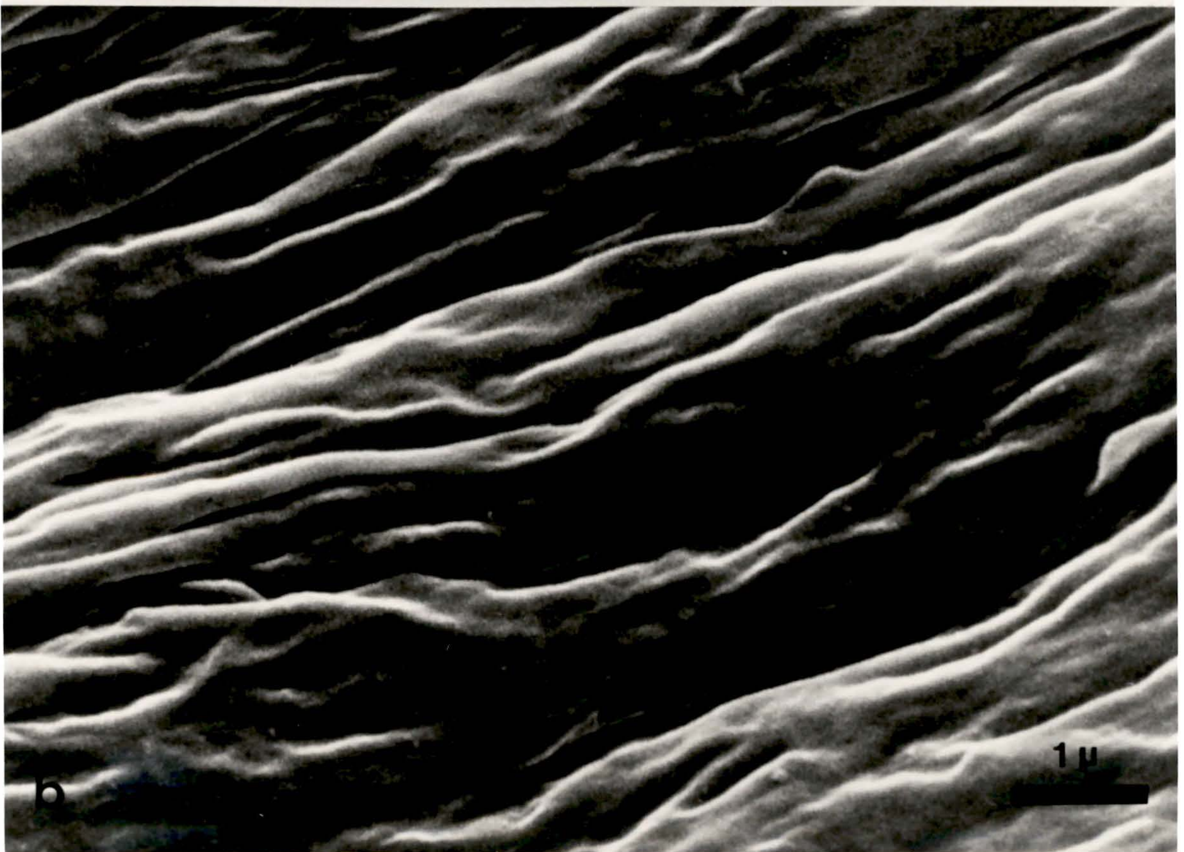

cut
mdl
hc
ge
10µ
c

Plate VII The cuticle lining of the second maxillary duct.

a) Scanning electron micrograph of thin cuticle lining of the maxillary duct. Surface is smooth with only slight irregularities and no apparent structural modifications. X 17,000.

b) As above, but from partially contracted region of maxillary duct, showing folds and ridges - resulting from contraction. X 17,000.


a
1 µ


b
1 µ

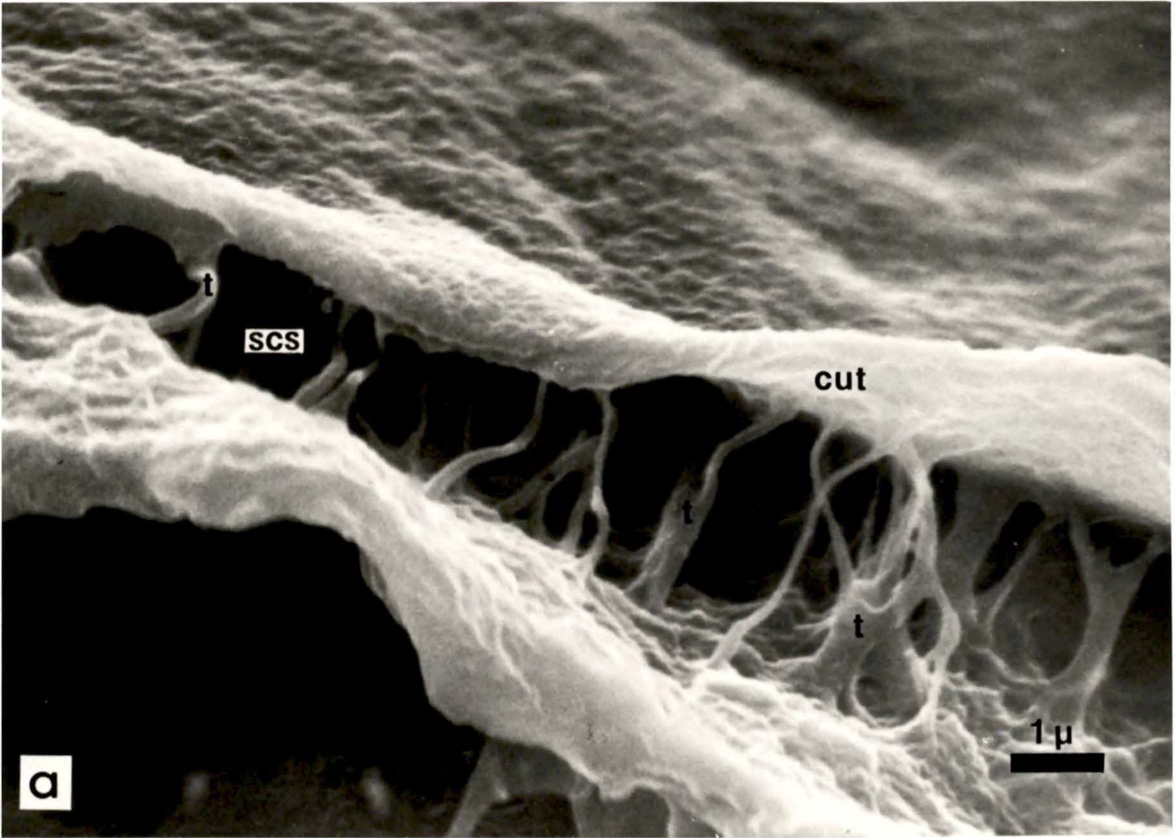
by elongate processes which appeared to be the only form of attachment between the two surfaces (Plate VIIa). These structures were accompanied by numerous shorter processes originating from the underlying tissue and only partially spanning the space between the tissue and the cuticle (Plate VIIIb). These latter processes were identified from transmission electron micrographs of this region as microvilli, while the longer processes or "trabeculae" appear to be the only points of attachment between the glandular epithelial cells and the overlying cuticle. It was noted during lengthwise slicing of formalin-fixed second maxillae, preparatory to S.E.M. examination of the maxillary duct, that the cuticle lining could be stripped away from the underlying glandular epithelium easily and cleanly, without apparent damage to either component, suggesting that the cuticle and epithelial tissue are only loosely interconnected. The explanation for this observation seems to be that the cuticle and underlying epithelium are connected by numerous short processes (the trabeculae) which extend across a narrow fluid-filled space separating most of their adjacent surfaces. Hence stripping away the cuticle involves merely breaking these processes, rather than peeling apart two closely applied and adhering surfaces.

The spacial relationships between the cuticular duct lining and the underlying epithelial cells and cell processes are particularly apparent in transmission electron micrographs of thin epon sections cut perpendicular to the duct wall (Plate IX), which confirm the earlier interpretation. The cuticular lining of the maxillary duct varies in thickness from one to several microns, and is anchored to the underlying epithelium at intervals by elongate cell processes (trabeculae), but is separated from these cells to form a

Plate VIII The maxillary duct subcuticular space.

a) Scanning electron micrograph of cut edge of maxillary duct, demonstrating the subcuticular space (scs). This space separates the cuticle from the underlying epithelial layer, and is spanned by numerous processes or trabeculae (t) which extend from the surface of the epithelial cells. X 12,000.

b) A similar preparation from a more proximal part of the maxillary duct reveals, in addition to the trabeculae, numerous microvilli (mv) extending into the subcuticular space from the apical plasma membrane of the epithelial cells. X 14,000.


t
scs
cut
t
t
1 µ
a

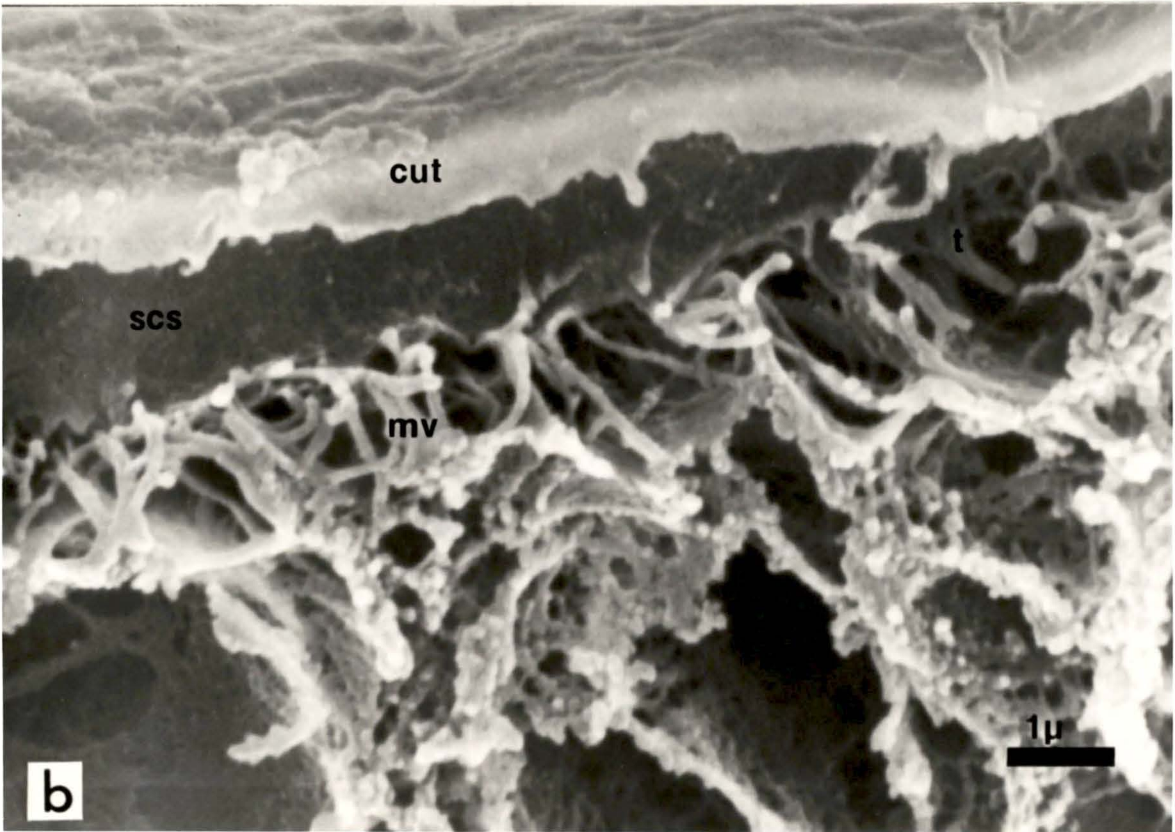

cut
t
scs
mv
1µ
b

Plate IX Ultrastructure of the maxillary duct wall. The cuticular lining of the duct (cut) is surrounded by a subcuticular space, into which trabeculae and numerous microvilli extend from the underlying epithelial cell layer. Extensive intercellular spaces (is) are also apparent beneath this cell layer. X 13,800.

is
mdl
cut
mv
1μ

narrow space of varying width into which numerous microvilli extend from the cells as modifications of the apical plasma membrane. The outer (adlumenal) surface of the cuticle is clearly marked by a thin lamina and numerous granules, both of dense-staining materials, while the inner (adepithelial) boundary is much less distinct (Plates X, XI). This arrangement of thin cuticle overlying epithelial cells with well developed apical microvilli is characteristic of cuticularized transporting epithelia in some arthropods, and is particularly reminiscent of the ultrastructure of insect (*Calliphora*) rectum and crab (*Callinectes*) gill, both of which are concerned with ion and water transport across thin cuticle (Berridge and Oschman, 1972). The abundance of mitochondria in the apical regions of the cell (Plate XI) and the extensive system of microtubules extending from the microvilli into adjacent portions of the cell (Plate XII) are further indications of a probable transport function, as are also the numerous intercellular spaces which often appear to be lined with glycocalyx (arrows, Plate XI) (Berridge and Oschman, 1972). These authors note that an abundance of mitochondria in epithelial cells is particularly indicative of active transport across that tissue, as they are required to carry out the oxidative phosphorylation and production of ATP necessary to drive the transport mechanisms.

The glandular epithelium surrounding the maxillary duct is seen by transmission electron microscopy to be composed of at least two and possibly three distinct cell types. The first of these forms a layer immediately below the cuticle and gives rise to the trabeculae and microvilli as already described. The cytoplasm of these "Type I" cells appears more densely-staining than that of the other cell types, and they

Plate X Organization of the glandular epithelium.

The glandular epithelium is composed of several types of cells (see text for further details), often separated along much of their adjacent borders by extensive intercellular spaces (is), which appear to be lined with glycocalyx (arrows). X 18,800.

cut - cuticle lining of maxillary duct; mdl - maxillary duct lumen; mit - mitochondria; mt - microtubules; mv - microvilli; t - trabeculae; v - vesicle or tubule systems surrounded by rough endoplasmic reticulum.

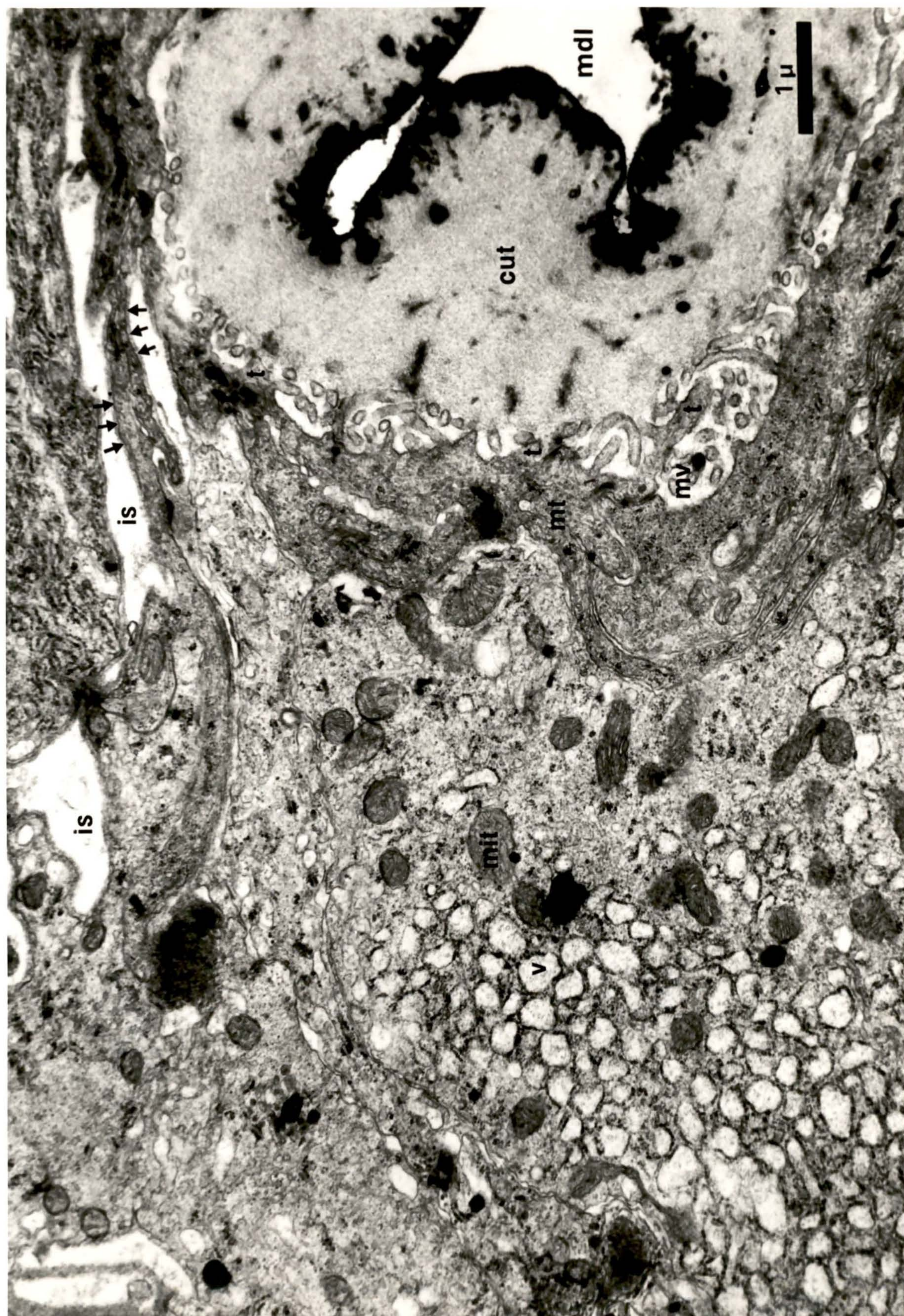

mdl
1 μ
cut
t
t
t
my
mt
is
is
mit
v

Plate XI Type I cells of the subcuticular epithelial layer.

Type I cells give rise to the microvilli (mv), are separated from other cell types by extensive intercellular spaces (is) lined with glycocalyx (arrows), and are characterized by a dense-staining cytoplasm containing microtubules and usually numerous mitochondria (mit). Type I cells are joined to one another in the apical regions by an occluding zonule (oz), and elsewhere by desmosomes (d). X 25,600.

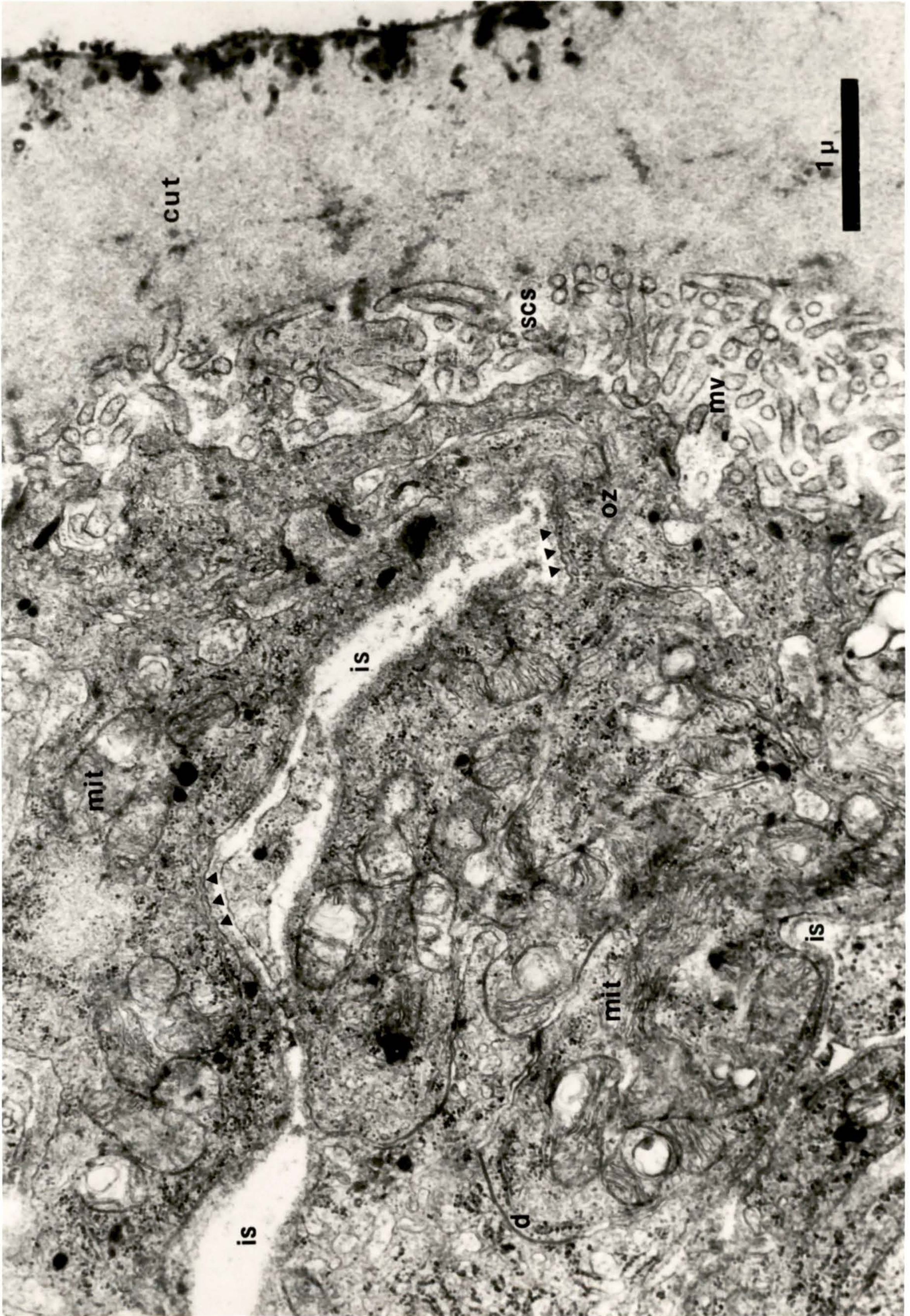
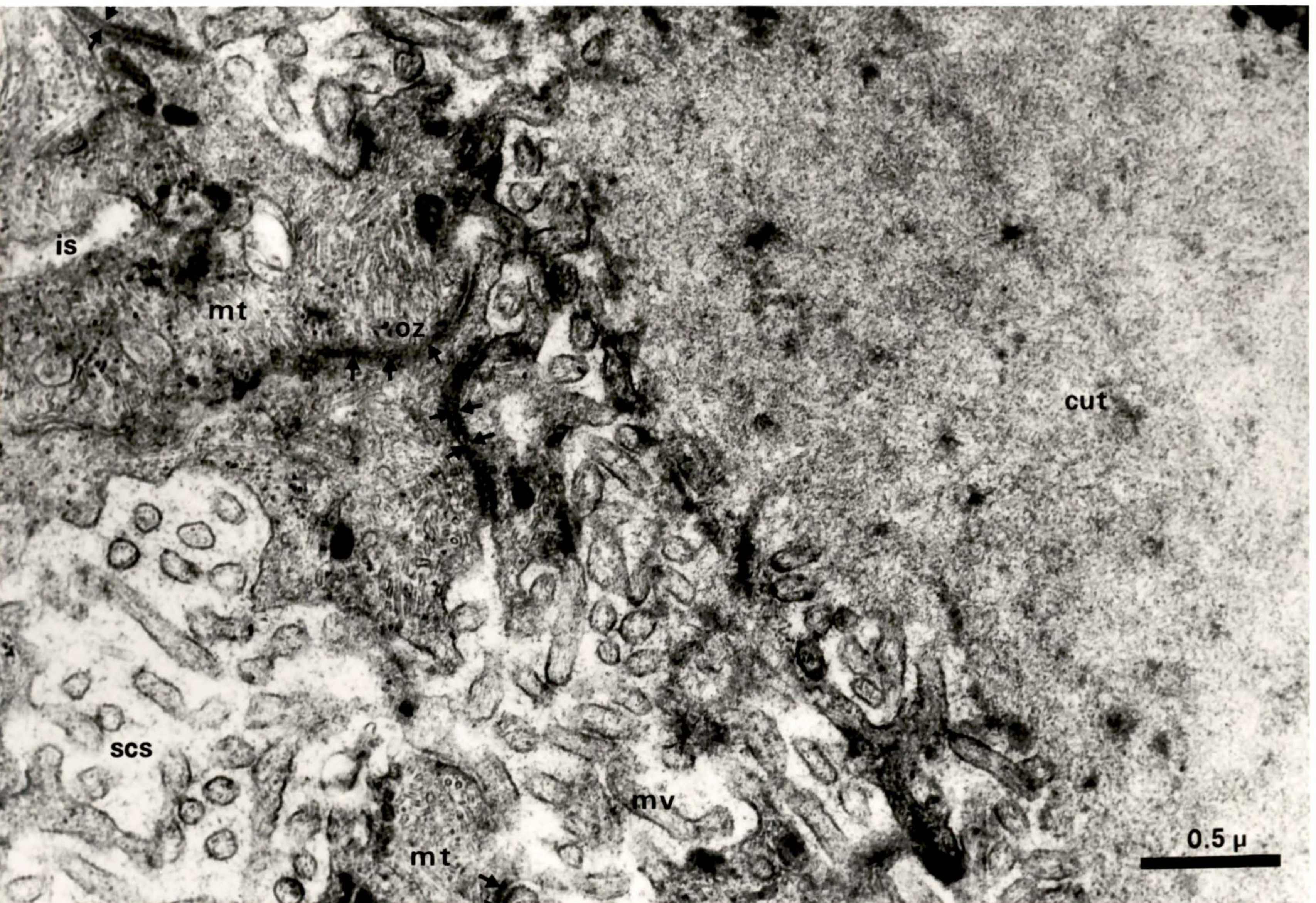

cut
1 µ
scs
mv
oz
is
mit
is
mit
is
d

Plate XII The apical region of Type I cells.

An extensive system of microtubules (mt) extending between the microvilli and the rest of the cell is a prominent feature of Type I cells. Occluding zonules (oz, arrows) are also common in the apical region and serve to prevent diffusion of materials between the intercellular (is) and subcuticular (scs) compartments by forming a continuous seal between adjacent cells. X 47,000.



is
mt
oz
cut
scs
mv
mt
0.5 μ

are almost completely separated from dissimilar cells, and sometimes also from one another, by an extensive system of wide glycocalyx-lined intercellular spaces or canaliculi (Plates IX, XI). Type I cells appear to be anchored to each other along their lateral surfaces primarily by separate desmosomes. Adhering zonules or desmosomes also occur near the apical microvillar surface of adjacent cells (arrows, Plates X, XII), though the intercellular space is typically sealed near this surface by an occluding zonule which presumably serves to prevent uncontrolled movement of water and ions by diffusion between the intercellular space and the subcuticular space.

A second layer of cells, lying peripheral to the Type I cell layer, is characterized by a less dense staining cytoplasm, an abundance of rough endoplasmic reticulum, and a basal plasma membrane developed into a complex system of canals and tubules, here termed a "basal labyrinth". As previously described, this layer is largely separated from the Type I cell layer by an extensive system of intercellular spaces, bridged periodically by septate desmosomes,and consists of cells apparently of at least two different types, though these may in fact represent only different phases of a single cell type. The two cell types differ mainly in the organization of the rough endoplasmic reticulum and development of the basal labyrinth, and differences also occur in the distribution of inclusions such as lipid droplets.

In cells here defined as belonging to Type II, the rough e.r. is not organized into tubule or vesicle systems and the basal plasma membrane forms an elaborate tubule system extending throughout much of the cell(Plates XIII, XIV. In addition, structures resembling lipid droplets, some-

times of quite considerable size, are commonly observed. These occur in two forms: dense staining, frequently large bodies (Plate XVa) which appear to grade into the surrounding rough e.r. at the margin (perhaps indicating synthesis and deposition or possibly resorption), and less dense staining, usually smaller bodies with more distinct margins (Plates XIVa, XVb). These differences in staining and morphology suggest synthesis and at least temporary storage of two different types of intermediate metabolites, though this possibility has not been investigated further in this study.

Cells of Type III are characterized by organization of the rough e.r. into extensive tubule or vesicle systems which occupy almost the entire cell (Plates X, XVI). The basal labyrinth of Type III cells is either greatly reduced or is incorporated within the rough e.r. - tubule system in such a way that the spaces within this system are continuous with the extracellular space beyond the basal plasma membrane. Lipid droplets and other inclusions were not observed in cells of this type.

Occasionally yet another type of cell was observed which contained numerous mitochondria and a moderately developed basal labyrinth, but little or no rough e.r. (Plates XVI, XVII). It is suggested for the moment that this latter cell type may simply represent an early stage of development of either a Type II or possibly a Type III cell, rather than another distinct cell type.

In summary, the Type I cells of the maxillary duct epithelium appear to possess all the ultrastructural features characteristic of a transporting epithelium, and the presence of lipid deposits and abundant rough e.r. in the Type II and Type III cells suggests an additional capability to synthesize and store or secrete lipids and proteins. Assuming

Plate XIII Type II cells of the maxillary duct epithelium.

a) Electron micrograph of oblique section through the basal region of a Type II cell. These cells characteristically contain abundant rough endoplasmic reticulum (rer) and have the basal plasma membrane modified into an extensive system of tubules, the basal labyrinth (bl), which occupies the basal region and also extends to other parts of the cell. Lipid droplets (l) are also frequently present. X 13,800.

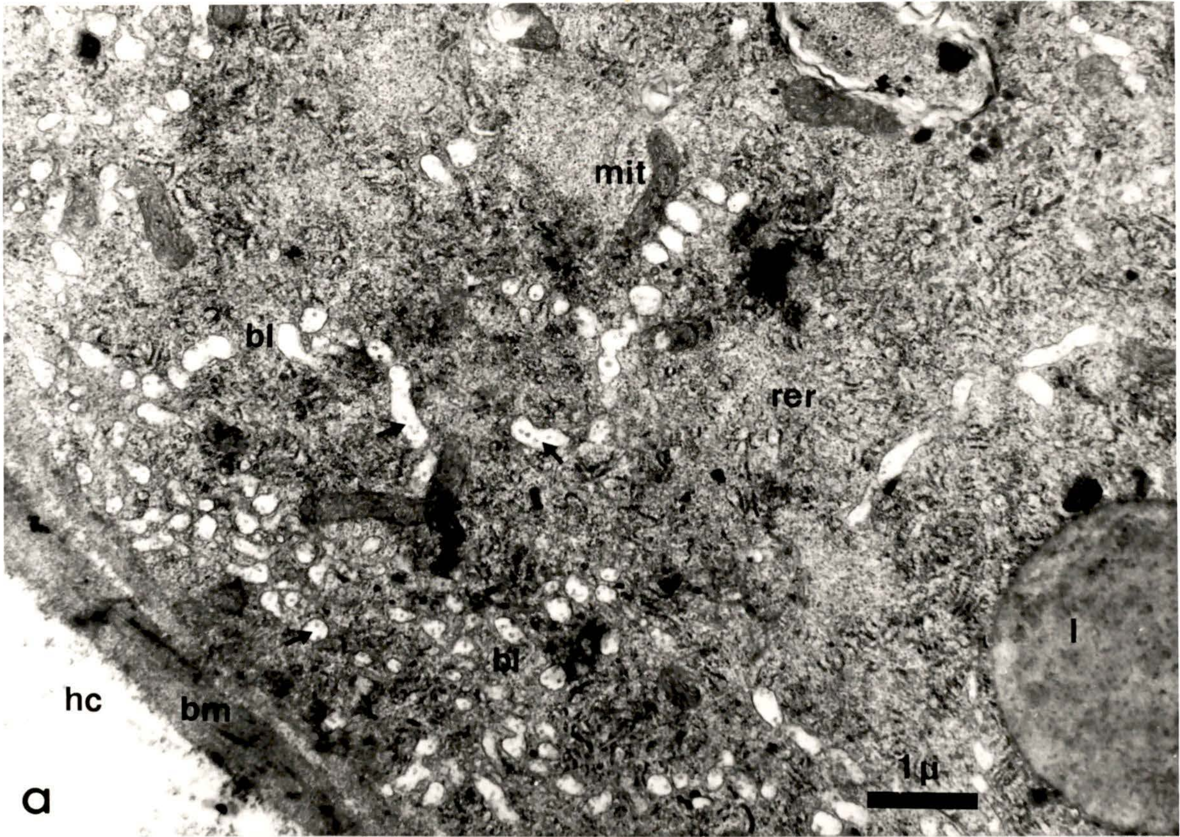
b) Electron micrograph of transverse section perpendicular to basement membrane (bm) at higher magnification than in a), showing rough e.r., basal labyrinth and openings of basal labyrinth to extracellular space via pores in basal plasma membrane (arrows). Note that this extracellular space is continuous with the haemocoel (h via the basement membrane. X 25,500.

hc
rer
bl
n
rer
bm
bl
l
hc
bl
b
0.5 µ
bm
a
1 µ

Plate XIV Evidence for metabolite synthesis and secretion in Type II cells.

a) Electron micrograph of basal region of Type II cell with well-developed basal labyrinth (bl), lipid droplet (l), rough e.r. (rer) and mitochondria (mit). Many of the tubules of the basal labyrinth seen in section show small granules (arrows) averaging about 300 Angstroms (0.03μ) in diameter in the lumen, which strongly imply secretion of material from the basal labyrinth of Type II cells into the haemocoel (hc). X 13,800.

b) Eletron micrograph of basal region of Type II cell with numerous mitochondria (mit) and organization of rough e.r. (rer) into parallel sheets in region of the nucleus (n), suggesting active protein synthesis. X 21,000.


mit
bl
rer
bl
l
hc
bm
1 µ
a

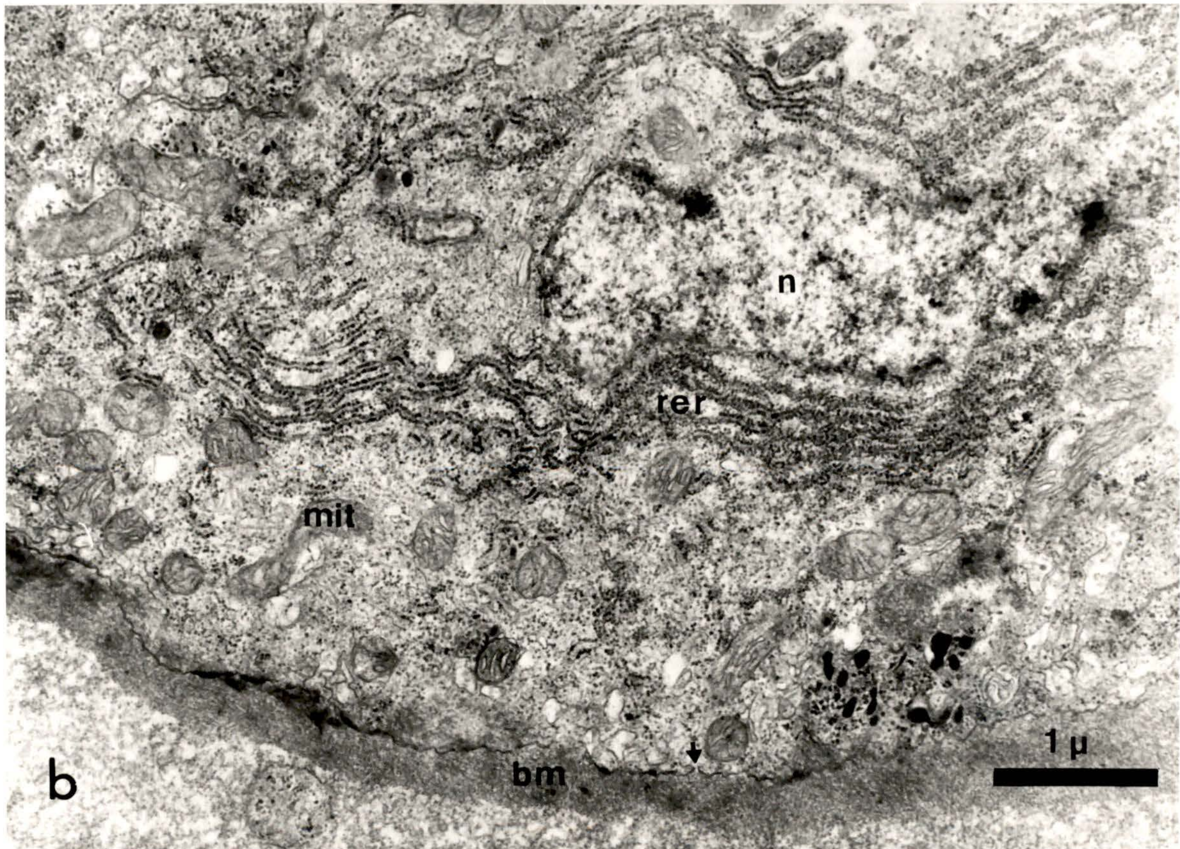
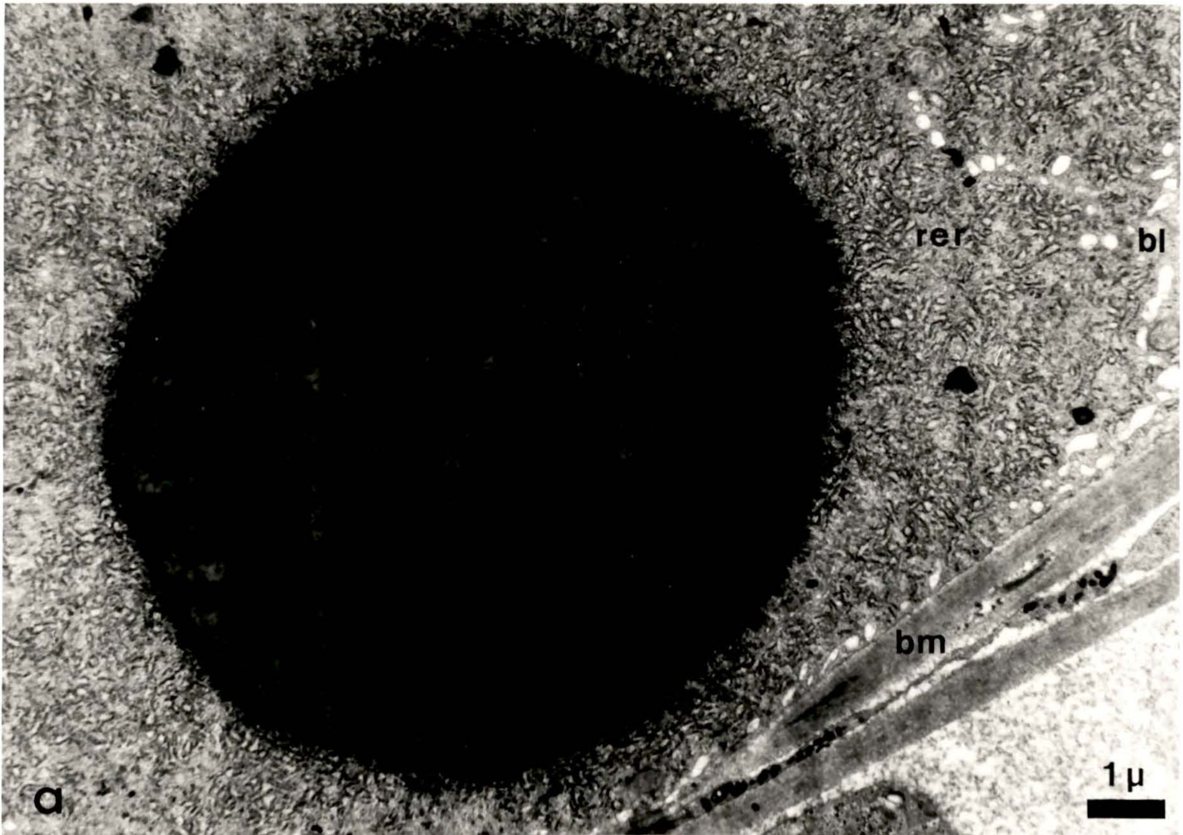

n
rer
mit
bm
1 µ
b

Plate XV Lipid droplets in Type II cells.

a) Electron micrograph of basal region of Type II cell containing abundant rough e.r. (rer) and a large dense staining lipid droplet about 9μ in diameter. The basal labyrinth is present but not extensively developed. X 10,000.

b) Electron micrograph of Type II cell containing rough e.r. (rer) organized into numerous small vesicles, mitochondria, and a lipid droplet (1) of a type much smaller, less dense staining and with a more clearly defined boundry than that seen in a). X 13,800.


rer
bl
bm
1 μ
a

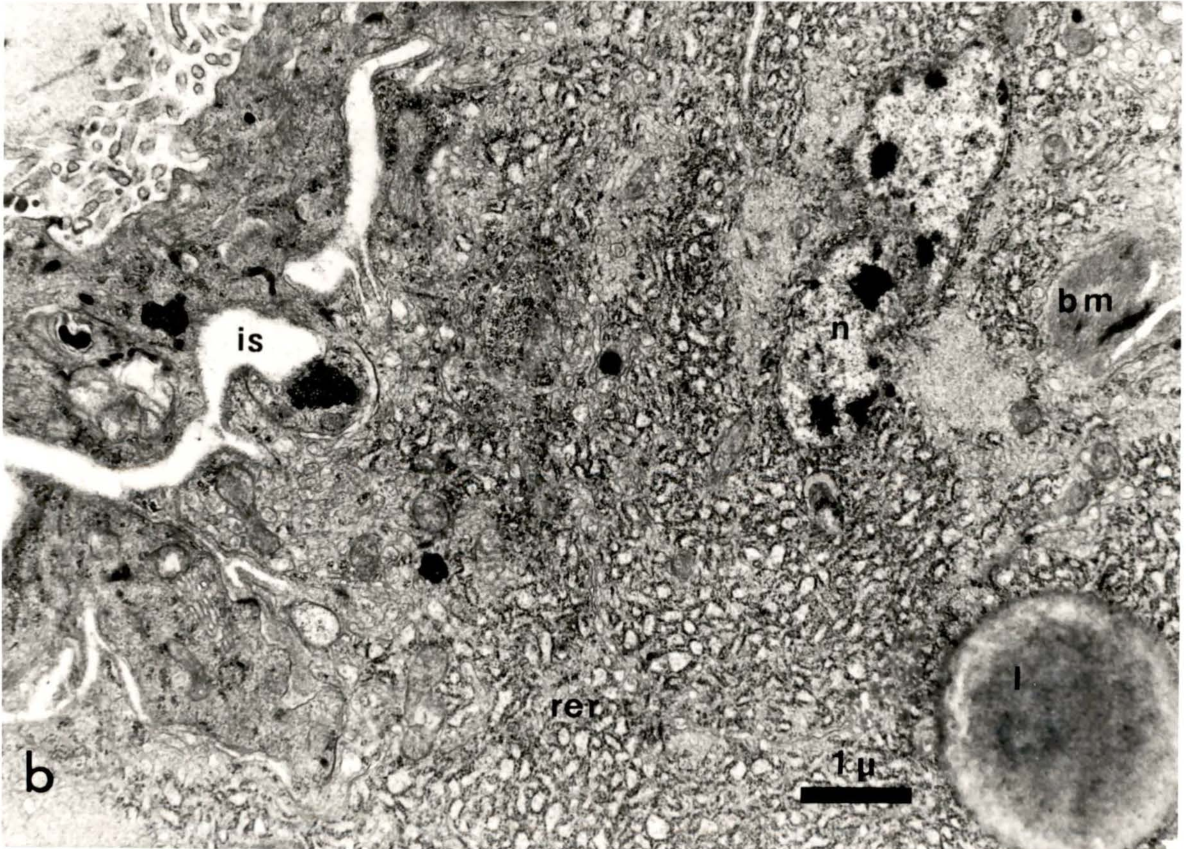

is
n
bm
rer
l
1 μ
b

Plate XVI Type III cells of maxillary duct epithelium.

Electron micrograph of region of maxillary duct epithelium showing examples of all structural types of cell encountered in this study (indicated by Roman numerals). The Type III cell contains abundant rough e.r. organized into large vesicles or tubules (v) which occupy almost the entire cell, while mitochondria are uncommon, lipid droplets are absent, and the basal labyrinth is either poorly developed or quite possibly incorporated into the vesicle system. In the latter event, type II, III, and IV cells could represent different stages of development and maturation of a single cell type. X 19,700.

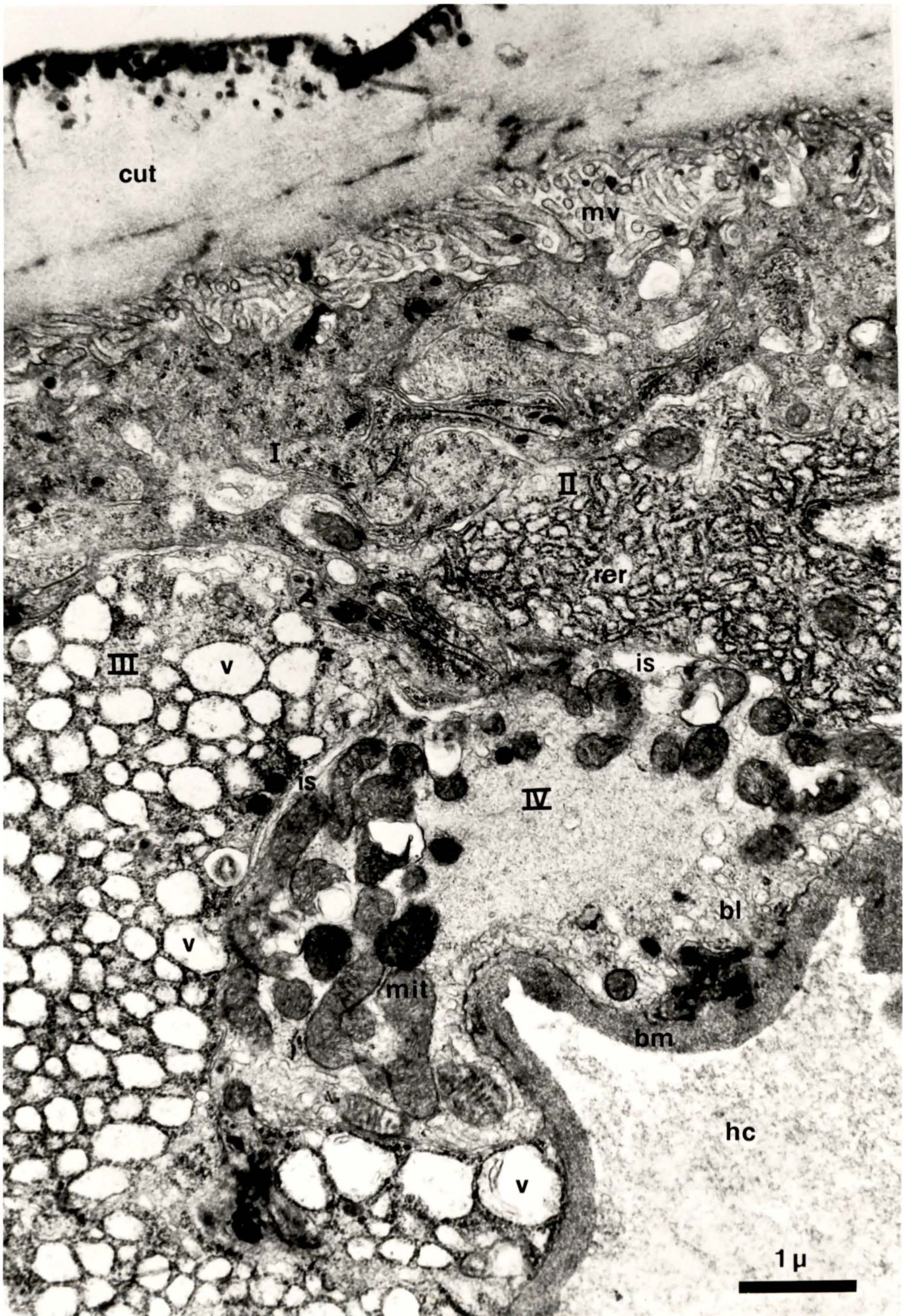
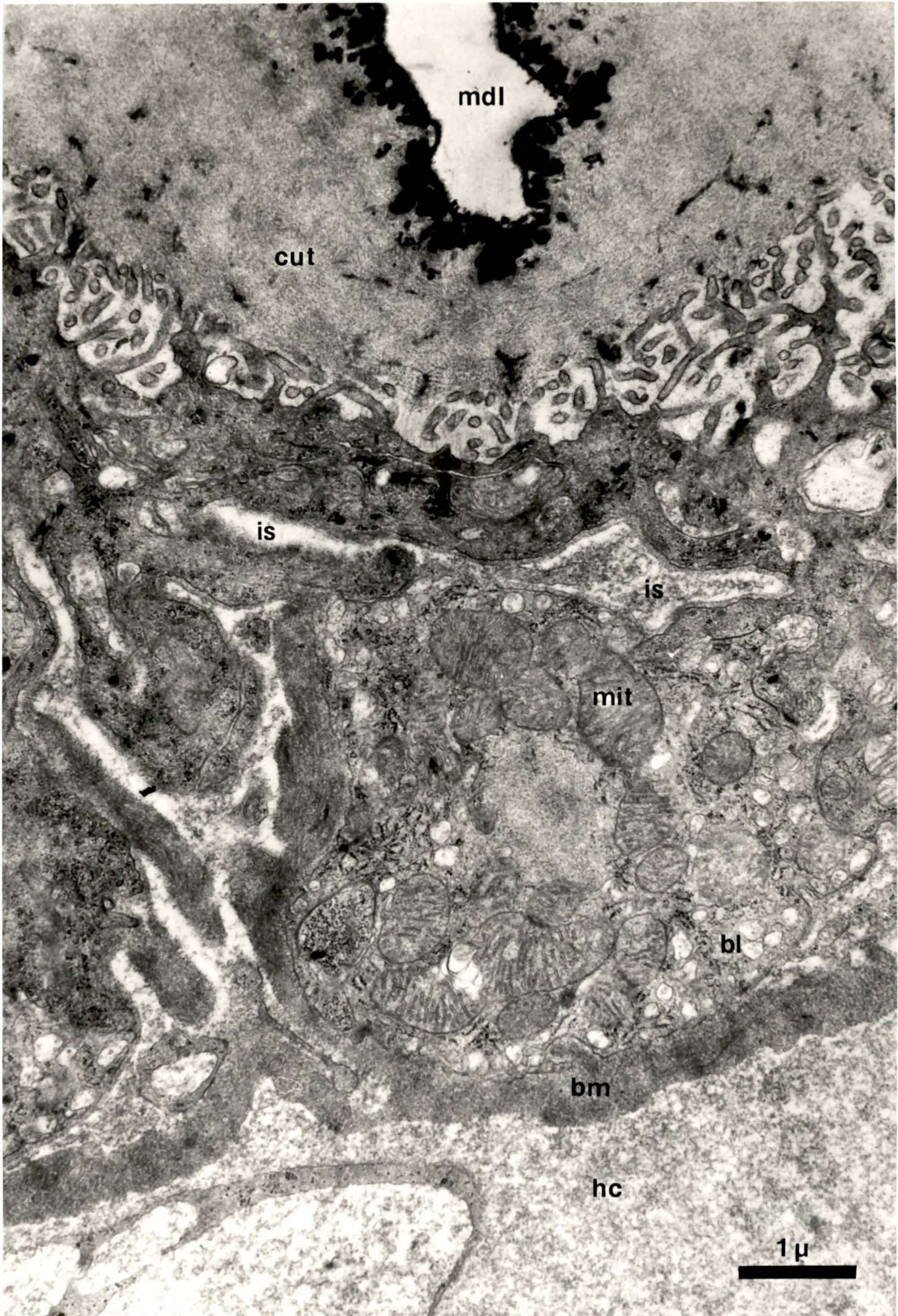

cut
mv
I
II
rer
III
v
is
is
IV
v
bl
mit
bm
hc
v
1 µ

Plate XVII Type IV (?) cells of maxillary duct epithelium.

Electron micrograph of Type IV (?) cell, containing many large closely packed mitochondria (mit), very little rough e.r. and a moderately developed basal labyrinth. X 20,000.


mdl
cut
is
is
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1 μ

that the necessary gradiants for movement of ions and water are produced along the microvilli and/or lateral plasma membranes of the Type I cells, a wide variety of low molecular weight compounds might be drawn by entrainment or solvent drag across the microvillar border and released into the extensive intercellular spaces. The Type II and Type III cells might then fulfill the logical function of regulating entry of these substances into the haemolymph of the parasite, by selectively absorbing the raw materials, passing some on to the haemolymph directly and incorporating others into intermediate metabolites either for temporary storage or release as needed to the haemolymph. While this hypothesis has not yet been verified experimentally, it follows logically from the currently accepted functions of the various tissue and cellular components as they are arranged within the epithelium about the second maxillary duct. Alternate hypotheses involving synthesis and secretion of materials into the host via the second maxillary ducts and bulla, particularly in opposition to the demonstrated route of uptake of inorganic ions and other metabolites, seem far less likely within the framework of our present understanding of host-parasite relations between Salmincola and its host fish. As yet there is no evidence to suggest a movement of materials from parasite to host, although this possibility cannot of course be completely eliminated.

(iii) The Proximal Maxillary Duct

In addition to the large maxillary duct already described, a second small cuticle-lined duct occurs near the base of the second maxilla, communicating with the saccular enlargement of the larger duct at its proximal end via a cuticular funnel. The funnel curves medially and the narrow duct passes through the base of the maxilla, curves to enter the

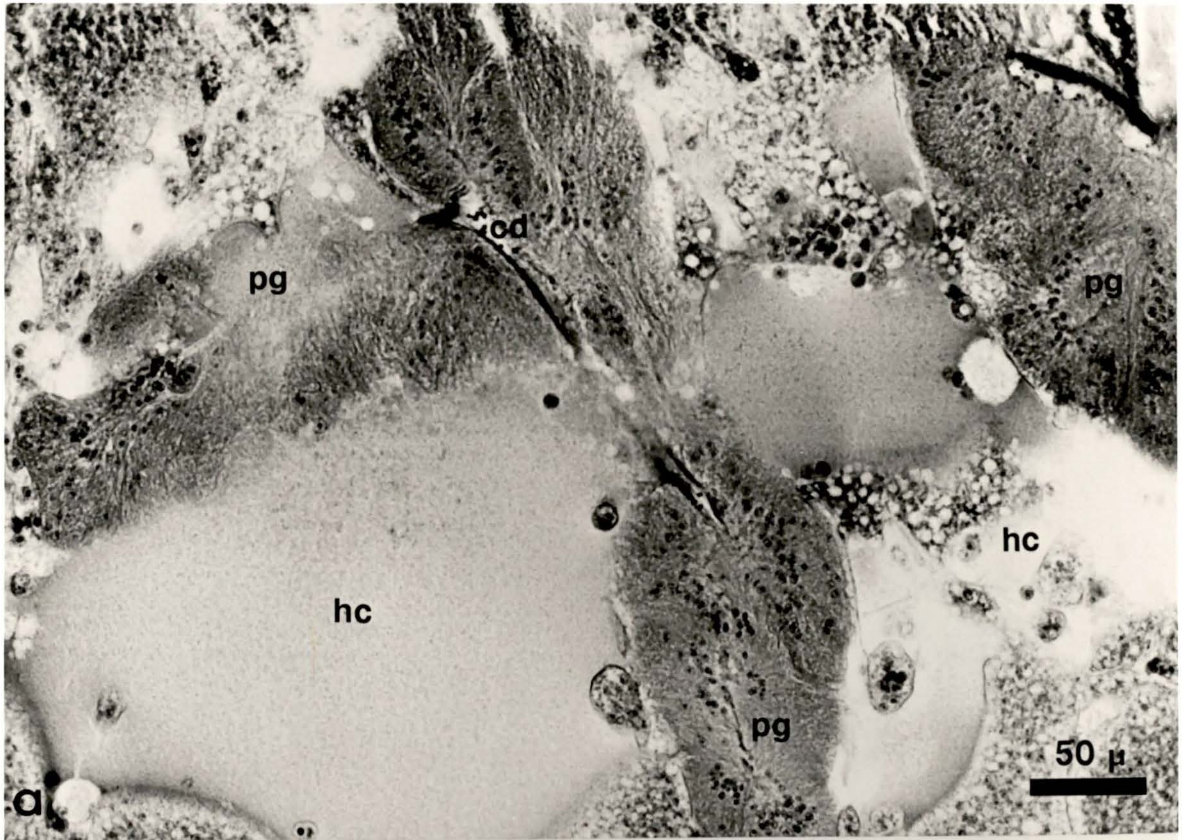
trunk, and continues posteriad just beneath the cuticle of the dorso-lateral trunk wall to terminate in a series of small lobular glands situated along the lateral walls of the trunk haemocoel. This structure, hereafter termed the proximal maxillary duct, is obscure and difficult to observe in the live animal, and is best demonstrated and observed by the method which led to its accidental discovery: injection of a vital dye solution (methylene blue or neutral red) into the lumen of the larger maxillary duct under pressure. This was accomplished using a small syringe fitted with a fine-drawn glass capillary, the tip of which was inserted tightly into one of the narrow ducts of the manubrium of the bulla after piercing the subanchoral surface. A considerable pressure is required, since the proximal duct is neither contractile nor elastic and expandable, unlike its larger counterpart, and is filled with a clear fluid which can only be displaced by the dye solution after rupture of one or more of the terminal glands in the haemocoel, at which point dye is observed to suddenly fill the narrow tubule and escape into haemocoelomic fluid.

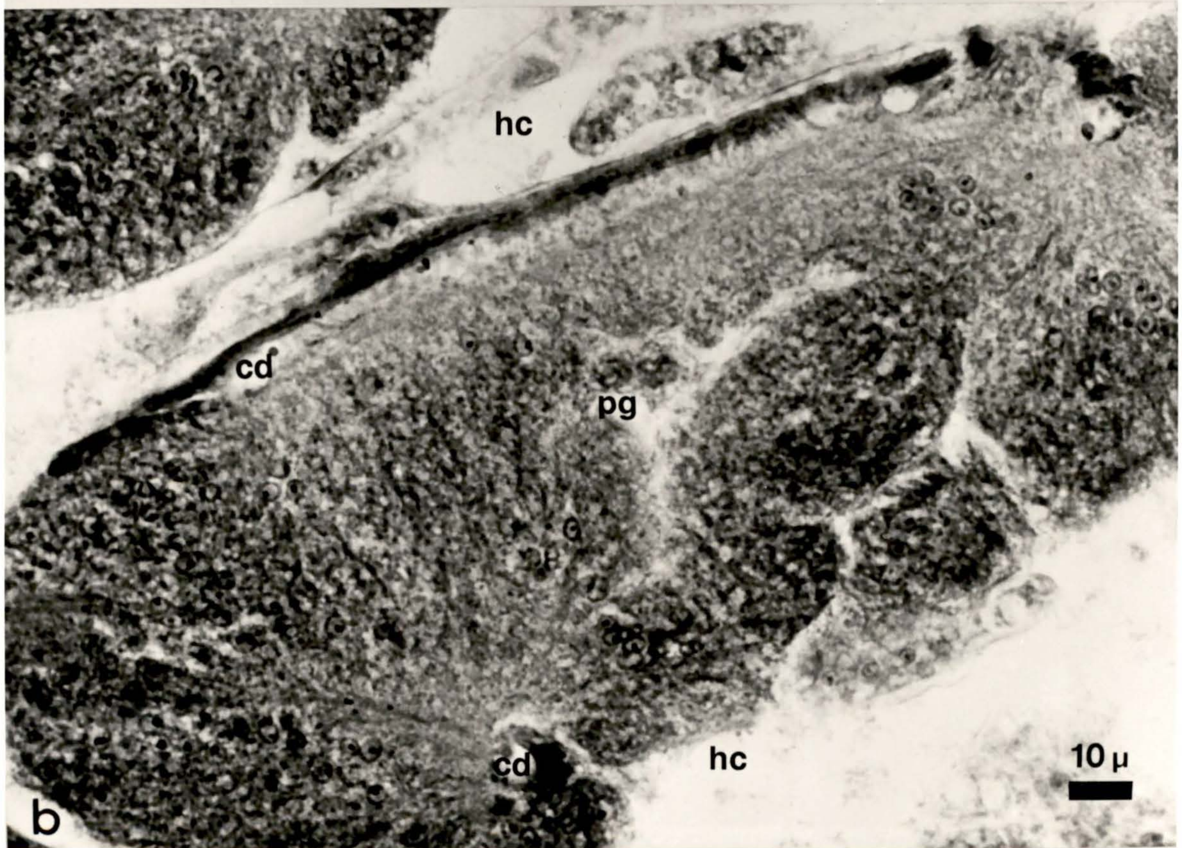
As seen in stained paraffin sections with the light microscope, the structure of the proximal duct appears to be a simple narrow cuticle-lined tube, surrounded by an equally simple epithelium of thin flat cells with sparsely distributed nuclei. The small terminal glands are variable in number and consist of one to several lobes composed of cells with ill-defined inter-cellular boundaries and prominent nuclei, radially arranged about a narrow central tubule (Plate XVIII). These tubules join to form the proximal duct, and are presumed to function in transfer of secreted fluid from the glands to the proximal duct, which in turn carries the

Plate XVIII Terminal glands of the proximal maxillary duct.

a) Photomicrograph of three lobes of the proximal maxillary glands (pg), lcoated laterally in trunk haemocoel (hc) of the copepod. The central two lobes are connected by a narrow collecting duct (cd), a number of which join to form the proximal maxillary duct. X 300.

b) Photomicrograph of single lobe of proximal gland, showing radial arrangement of cells about the collecting duct in each lobe. The collecting duct typically runs along the surface of a lobe of gland tissue rather than through its centre, as indicated in the upper portion of the figure. X 800.


cd
pg
pg
hc
hc
pg
50 µ
a


hc
cd
pg
cd
hc
10 µ
b

secretion to the lumen of the larger maxillary duct. The fine structure of the proximal duct and glands was not examined in this study.

## II. Operation of the Bulla-Maxillary Duct System: Observations

Frequent contractions of the second maxillae by means of the longitudinal muscle bands may be observed almost continually in any healthy _S. californiensis_, whether attached to its host fish, detached and immersed in either saline or distilled water, or mounted in a capillary tube. The maxillae contract in sequence rather than in unison, the contractions alternating regularly from side to side and causing the copepod (when anchored by the bulla) to sway back and forth in an arc about its point of attachment. Synchronized with each contraction of a particular maxilla is a contraction of the saccular portion of the large duct within that maxilla, combined with a corresponding relaxation and lengthening of the opposite maxilla and enlargement of its maxillary duct end-sac (Fig. 4). This results in a continuous back and forth movement of the fluid within the maxillary ducts from one maxilla to the other, via the connecting loop-duct within the bulla. These ducts thus form in combination a single long tubular compartment, essentially closed and with a contractile saccular reservoir at each end (since the proximal ducts entering the contractile end-sacs appear to play no part in this movement of fluid). The back and forth movements can easily be demonstrated by observing with a dissecting microscope a few granules of particulate carbon or a small air-bubble introduced into the bulla loop-duct with a fine-drawn glass capillary, either of which can be seen to oscillate back and forth with each maxillary contraction and resulting pulse.

In addition, movement of small inclusions (haemocytes) in the fluid within

Fig. 4 Diagrammatic sequence showing operation of the second maxillary "pump" (anterior view).

a) Intermediate stage, at rest.

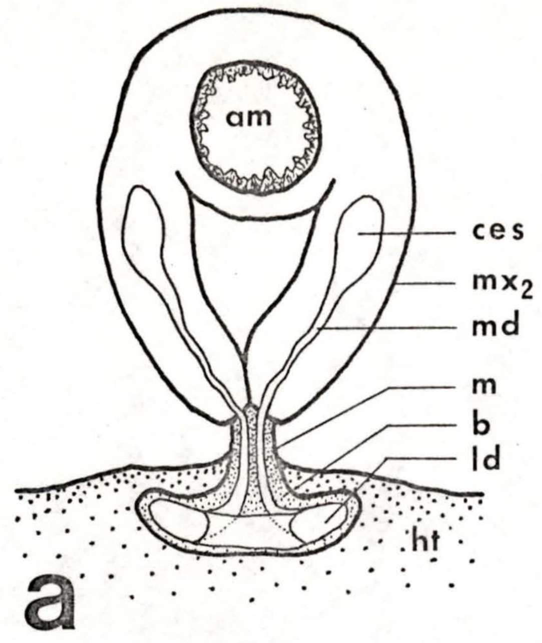
b) Contraction of left maxilla and maxillary duct end sac, extension of right maxilla and expansion of right duct end sac.

c) Intermediate stage; relaxation of left maxilla and some return of fluids, distension of right maxilla reduced.

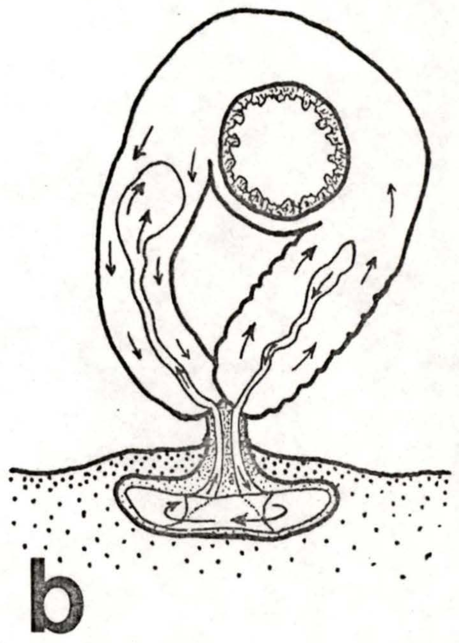
d) Contraction of right maxilla and maxillary duct end sac, extension of left maxilla and expansion of left duct end sac. The cycle is repetitive and continuous.

am - anterior midgut; b - bulla; ces - contractile end sac of maxillary duct; ht - host tissue surrounding bulla; ld - loop duct of bulla; m - manubrium of bulla; md - second maxillary duct; $mx_2$ - second maxilla. Arrows indicate movement of haemal and maxillary duct fluids.

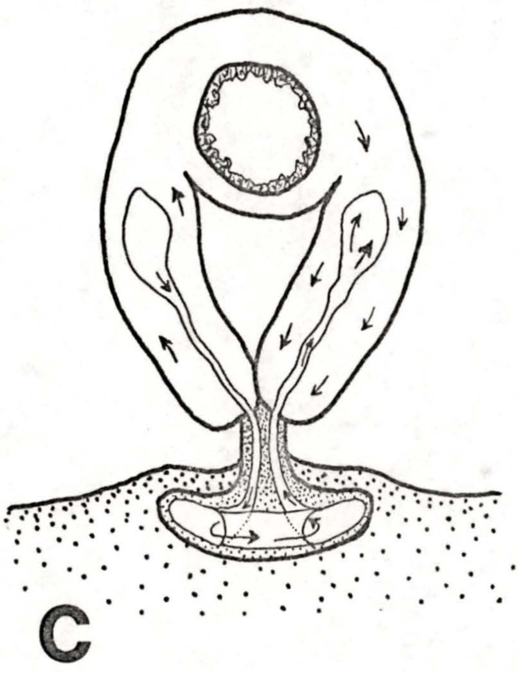
4



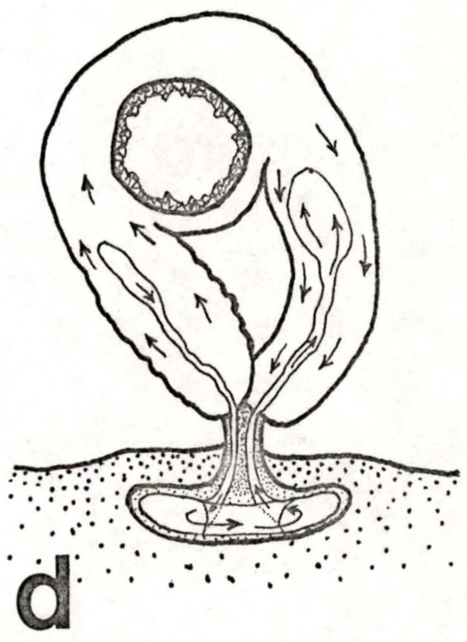

a



b



c



d

the haemal spaces of the second maxillae (between gland tissue surrounding the duct and the outer wall) can be observed through the transparent cuticle. These movements indicate a flushing of haemal fluid back and forth between the maxillary haemal spaces and the trunk haemocoel with each contraction and subsequent extension of the maxilla. There are indications of directionality of flow of haemal fluid leaving the maxillae such that the bulk of this fluid passes anteriorly towards the region of the mouthparts and circumoesophageal ganglion. These observations suggest the possibility (as yet unconfirmed) of existence of a system of valves at the bases of the second maxillae which may at least partially control the movement of haemal fluid entering and leaving the maxillary haemal spaces.

The second maxillae and bulla together may thus be regarded as a two-stage reciprocating pump, the power being supplied by alternate contraction of the muscles of opposing maxillae and duct end-sacs. One stage, represented by the ducts of the second maxillae and bulla, forms a closed tubular compartment in which a fixed volume of fluid is contained and shunted back and forth via the loop-duct of the bulla. The second stage is represented by the haemal spaces of the maxillae and any valves which may exist at their bases, these forming open compartments communicating with the anterior trunk haemocoel from which haemal fluid is repeatedly withdrawn and returned. (See Appendix I for a general discussion of circulation of haemal fluid in _S. californiensis_.)

Assuming for the moment that the sole functions of the bulla and second maxillae were attachment and orientation of the copepod, the latter stage of the "maxillary pump" might be considered to function only in providing adequate irrigation of the haemal spaces of the maxillae as a result of orienting movements by contraction and flexion of these appendages. However, the nature

of the contractile reservoirs, maxillary ducts and the interconnecting loop-duct as a closed tubular compartment in which a fixed fluid volume is constantly shunted back and forth via the bulla by regular alternating contractions strongly suggests that the contained fluid may act as a carrier for one or more substances passing between parasite and host across the bulla. This hypothesis is further supported by the highly porous reticular fine structure of the bulla matrix, though a direction of travel for such a substance is not directly indicated by these structures.

However, it is apparent from incubation of S. californiensis in either distilled water or salmonid saline containing fluorescein (the water-soluble sodium salt) or methylene blue that the bulla is highly permeable to these compounds. Both enter and pass through the bulla matrix freely and diffuse into the fluid within the loop-duct. After a short period of time (a few minutes to about half an hour, depending on the rate of contraction of the second maxillae) these substances can be seen in the lumen of the cuticle-lined maxillary ducts. (This uptake of dye into the ducts is most easily observed with copepods mounted in capillary tubes containing a dye/saline solution to which only the bulla is exposed.) After transfer of the copepod to clear solutions containing no dye, the dyes can be observed leaking back out of the ducts via the bulla until the bulla, ducts and contained fluid again appear almost colourless. (Some residual staining of the duct walls or underlying tissue may occur with methylene blue, though this usually also disappears gradually.) Hence the bulla is clearly quite freely permeable in either direction to ions and molecules in solution up to a molecular weight of at least several hundred (fluorescein m. wt. = 376; methylene blue m. wt. = 320), and the contained fluid is capable of acting as a carrier for transporting these materials into the large ducts

of the second maxillae, where they are separated from the surrounding internal fluids of the parasite by only a thin layer of modified epithelial tissue which appears structurally capable of transport functions.

The fluid within the closed compartment of these ducts may thus be regarded as an extension of the internal tissue-fluid environment of the host fish, or at least those components of that environment capable of diffusing through the pellicle and matrix of the bulla. Availability of such materials to the parasite is then presumably determined by their ability to penetrate the thin cuticle lining of the maxillary ducts, together with any active or passive exchange processes which may occur across the surrounding modified epithelial tissues.

Dyes were never observed to enter the narrow proximal ducts, which could only be filled with dye solution by injection under pressure as previously described. The function of the proximal duct and the associated glands is uncertain, but its structure suggests a one-way transfer of secreted fluid, unlike that of its larger and more distal counterpart. It therefore seems likely that the glands and proximal duct are responsible for secretion and transport of the fluid which fills the maxillary ducts and loop-duct of the bulla. They probably function in the adult to replace any fluid lost from the closed duct compartment, either across the duct walls or into the host via the bulla, and may also be responsible for production of the fluid causing initial inflation of the bulla shortly after implantation and attachment.

III. Permeability Tests

A series of experiments was designed to indicate qualitatively the relative degrees of permeability of various external surfaces of adult female *S.*

Table IV. Summary of permeability tests with S. californiensis

| Test Substance | Cuticle | Penetration Bulla | Second Max. duct walls |
|---|---|---|---|
| $Ag^+$ | - | + | + (AgCl deposits formed) |
| $MnO_4^-$ | - | + | + (tissues oxidized) |
| Salmonid saline | - | + | + (prolonged survival) |
| $Mg^{++}$ | - | + | + (caused narcotization; rate conc.-dependant) |
| $CN^-$ | | + | + (rapid, lethal; rate conc.-dependant) |
| Neutral red | (+) | + | + (vital staining) |
| Toluidine blue | - | + | + (vital staining) |
| Methylene blue | - | + | + (vital staining) |
| Crystal violet | - | + | - |
| Janus green | - | + | - |
| Light green SF yellowish | - | + | - |
| Fast green FCF | - | + | - |
| Phenol red | - | + | + (vital staining) |
| Uranin (disodium fluorescein salt) | - | + | (+) (slow, inconsistent) |
| $^{14}C$-amino acids | | + | + ($^{14}C$ in body tissues) |
| $^{14}C$-glucose | | + | + ($^{14}C$ in body tissues) |
| Ronnel | - | ? | - (no toxicity) |
| Trolene 20L | - | ? | - (no toxicity) |
| Ruelene | +(toxic) | ? | - (no toxicity) |
| Ruelene 25E | - | ? | - (no toxicity) |
| Phenoxethol | +(toxic) | ? | - (no toxicity) |

*californiensis* to a range of types of compounds. The results follow and are summarized in Table IV.

(i) Ionic solutions

Permeability of the second maxillary duct linings to $Cl^-$ ion was clearly demonstrated by immersion of rinsed copepods in 0.01 M $AgNO_3$ solution and subsequent development of black deposits of silver, precipitated as AgCl by contact with $Cl^-$ ions escaping from the body fluids of the parasite. The ducts appeared intensely black due to the finely granular precipitate which was located within and immediately below the cuticle lining. Furthermore, the duct linings were the only external cuticular surfaces of any consequence which showed permeability to either $Ag^+$ or $Cl^-$ ion. Small deposits appeared at the tips of the first and second antennae and the small genital process, but the remainder of the external cuticle was negative, indicating impermeability to either $Ag^+$ or $Cl^-$ ions. In fact, the entire cuticle surface appeared hydrophobic, as if covered by a wax layer. (This was later confirmed by a positive reaction to Sudan black stain, indicating the presence of considerable amount of bound lipid, possibly including lipoprotein, in the outer layers of the cuticle of the body surface.) However, some leakage of chloride ion was noted via the small cement plugs sealing the fertilization ducts of the genital process after spermatophore implantation, and via the egg sac cement plugging the openings of the oviducts, these areas also accumulating black deposits.

Interestingly, in those copepods bearing egg sacs, the cement matrix between the eggs showed more dense silver deposits near the end attached to the parasite, the density decreasing with distance from the point of

attachment at the oviduct opening, while the eggs themselves showed no indication of a positive response. Silver deposits within the cement matrix could be prevented by removing the egg sacs from the copepod and incubating them in distilled water for 30 - 60 min. prior to treatment with $AgNO_3$ solution. These observations indicate that the egg membranes are impermeable to both inward diffusion of $Ag^+$ ions and outward movement of $Cl^-$ ions, and that the deposition of AgCl observed in the egg sac cement was due to $Cl^-$ ions leaking from the body fluids of the copepod via the permeable cement filling the oviducts. It is suggested that if the egg membranes are capable of active uptake of $Cl^-$ (and presumably also $Na^+$ and other ions) this leakage from the female's oviducts could well serve in providing a source of essential ions for the developing eggs, in which both nauplius larval stages are confined. If the copepod is capable of obtaining ions as necessary from the host via the bulla and second maxillae, such a mechanism might prove feasible energetically, since ions lost via the egg sacs could be readily replaced from the comparatively infinite pool of the host's tissue fluids.

Results with permanganate ion strongly supported those observed with $AgNO_3$ solution. In copepods with actively contracting second maxillae, the walls of the maxillary ducts rapidly (5-10 min.) acquired a "burnt" brown appearance due to oxidation of the epithelial tissue underlying the thin cuticle lining by $MnO_4$ ion, after which maxillary contractions gradually ceased. Again the only other cuticle surfaces which permitted permanganate ion penetration were the tips of the first and second antennae and the genital process, the remainder of the body surface showing no indications of permeability. The spermatophore duct plugs and egg sac

matrix were also penetrated and responded positively, but in this instance the permeable substance of both structures, as well as the matrix of the bulla and manubrium, reacted strongly with the $MnO_4^-$ ion, becoming oxidized throughout to a dark brown or black colour and brittle consistency.

Experiments with salmonid saline provided further evidence of the permeability of the maxillary duct walls to ions in solution, and demonstrated the bulla and maxillary ducts to be a major route of transfer of essential ions. Copepods incubated in distilled water were found to survive for a maximum of three days, generally becoming moribund within 24 to 36 hours, while copepods in salmonid saline routinely survived for seven to ten days, sometimes longer. This clearly indicates the ability of a saline solution to prolong survival time of Salmincola detached from its host, presumably by minimizing osmotic stress and perhaps also by providing an available source of essential ions in the absence of ths host's tissue fluids. Copepods incubated in isosmotic (0.30 M) sucrose solution showed no detectable differences in survival times from control animals incubated in distilled water, indicating that the prolonged survival observed with saline is dependent upon the presence of ions in the external medium, rather than the result of eliminating the osmotic pressure differences between the external medium and the body fluids of the copepod. Hence active uptake of ions from the saline solution into the haemal fluids or passive exchange of ions between these media is implied.

Development of the capillary tube/vial system for isolation of the bulla from the external medium permitted identification of the bulla and second maxillary ducts as the only portion of the body surface across which a

saline solution was effective in prolonging survival time. With the bulla exposed to either distilled water or isosmotic sucrose in the capillary tube no survival-prolonging effect was noted, regardless of whether the remainder of the external cuticle of the copepod was exposed to distilled water, isosmotic sucrose or salmonid saline. However, with saline in the capillary tube survival of the copepod was markedly extended, regardless of which solution was used as the external medium, as indicated by the results summarized in Table V.

Table V. Mean and range of survival times (in days) for *S. californiensis* mounted in capillary tubes and exposed to various combinations of distilled $H_2O$, isosmotic (0.30M) sucrose and salmonid saline as external and tube media (n=10).

| Capillary tube medium | External medium | | |
|---|---|---|---|
| | distilled $H_2O$ | sucrose (0.30M) | salmonid saline |
| dist. $H_2O$ | 2.6 (2-3) | 2.1 (1-3) | 2.4 (1-4) |
| Sucrose (0.30M) | 2.2 (1-3) | 2.7 (2-4) | 2.5 (2-3) |
| Salmonid saline | 13.5 (10-16) | 11.5 (8-14) | 11.0 (8-13) |

In addition, numerous copepods used as controls in subsequent experiments were kept alive *in vitro* using the capillary tube and saline method for periods of at least 12 - 14 days, with one instance of survival for 21 days. The saline solution to which only the bulla is exposed in these experiments substitutes for the tissue fluids of the host which bathe the bulla in nature. The dramatic increase in survival time of the copepod *in vitro* resulting from this exposure suggests a link between the internal environments of the host and parasite *in vivo*, via the bulla and maxillary

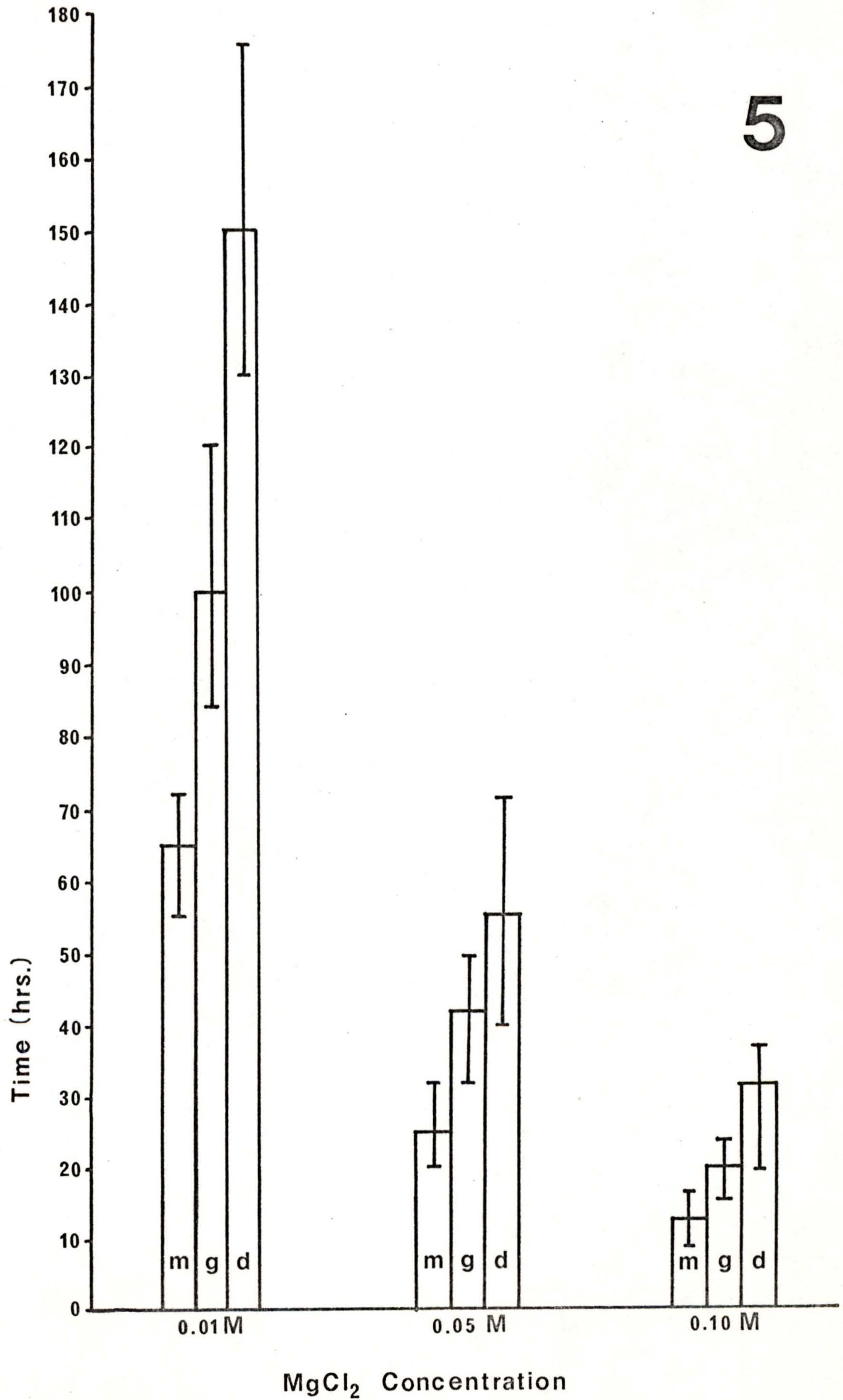
ducts. Since the bulla is an acellular non-living structure it is logical to assume that any exchanges occurring across its surfaces are purely passive, and that any active processes which might mediate exchange of material between host and parasite must therefore occur across the modified epithelium surrounding the maxillary ducts.

More compelling evidence for the existence of a pathway for uptake of dissolved ions via the bulla and maxillary ducts results from experiments with $MgCl_2$ solutions. The $Mg^{++}$ ion proved toxic to *Salmincola* when presented via the bulla and second maxillae and produced a fixed sequence of observable responses in the copepod. These were, firstly, onset of paralysis of the second maxillae and cessation of contractions, followed by suppression of normal gut movements (and hence circulation of the haemolymph), and finally death. The time interval required to elicit each of these responses was concentration-dependant, decreasing with increasing concentration of $Mg^{++}$ (Fig. 5). This suggests that the rate of influx of $Mg^{++}$ ions via the bulla and maxillary ducts is directly related to the concentrations available at the subanchoral surface of the bulla.

Control animals were mounted in capillary tubes containing solutions prepared as for the test groups but with equivalent concentrations of NaCl added rather than $MgCl_2$. These animals survived the duration of the experiments (up to seven days for the lowest $MgCl_2$ concentrations tested) without apparent ill effects or loss of activity. Copepods mounted in capillary tubes containing saline but exposed to similar concentrations of $MgCl_2$ in distilled water as the external medium also survived the seven-day period without visible effect, providing further evidence that

Fig. 5 Effects of absorption of $Mg^{++}$ via the bulla _in vitro_ (capillary tube system): exposure time for appearance of response vs. $MgCl_2$ concentration. (Vertical bar = mean, vertical line = range; n = 20.)

m - paralysis of second maxillae and cessation of pumping; g - paralysis of gut and cessation of haemal fluid circulation; d - death (cessation of all visible muscular activity).


5
Time (hrs.)
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
m
g
d
m
g
d
m
g
d
0.01 M
0.05 M
0.10 M
MgCl2 Concentration

cuticle of the external body surface is essentially impermeable to ions in solution. It thus appears that the bulla and maxillary ducts constitute the only pathway by which ions in solution are capable of entering the copepod in significant amounts.

(ii) Metabolic inhibitors

Saline solutions containing cyanide ion ($CN^-$) proved extremely toxic to S. californiensis when presented via the bulla and maxillary ducts. As with $Mg^{++}$, cessation of maxillary contractions was the first visible response, this occurring almost immediately upon contact at the higher concentrations used. At the same time or shortly thereafter the rhythmic motions of the gut became erratic, to be followed somewhat later by death. (A prolonged period of normal gut activity following maxillary paralysis as noted with $Mg^{++}$ ion was not observed.) Death as a result of $CN^-$ influx via the maxillary ducts occurred after exposure periods ranging from 2 - 3 minutes up to 36 hours, the times increasing with decreasing concentration of KCN in the capillary tube (Fig. 6).

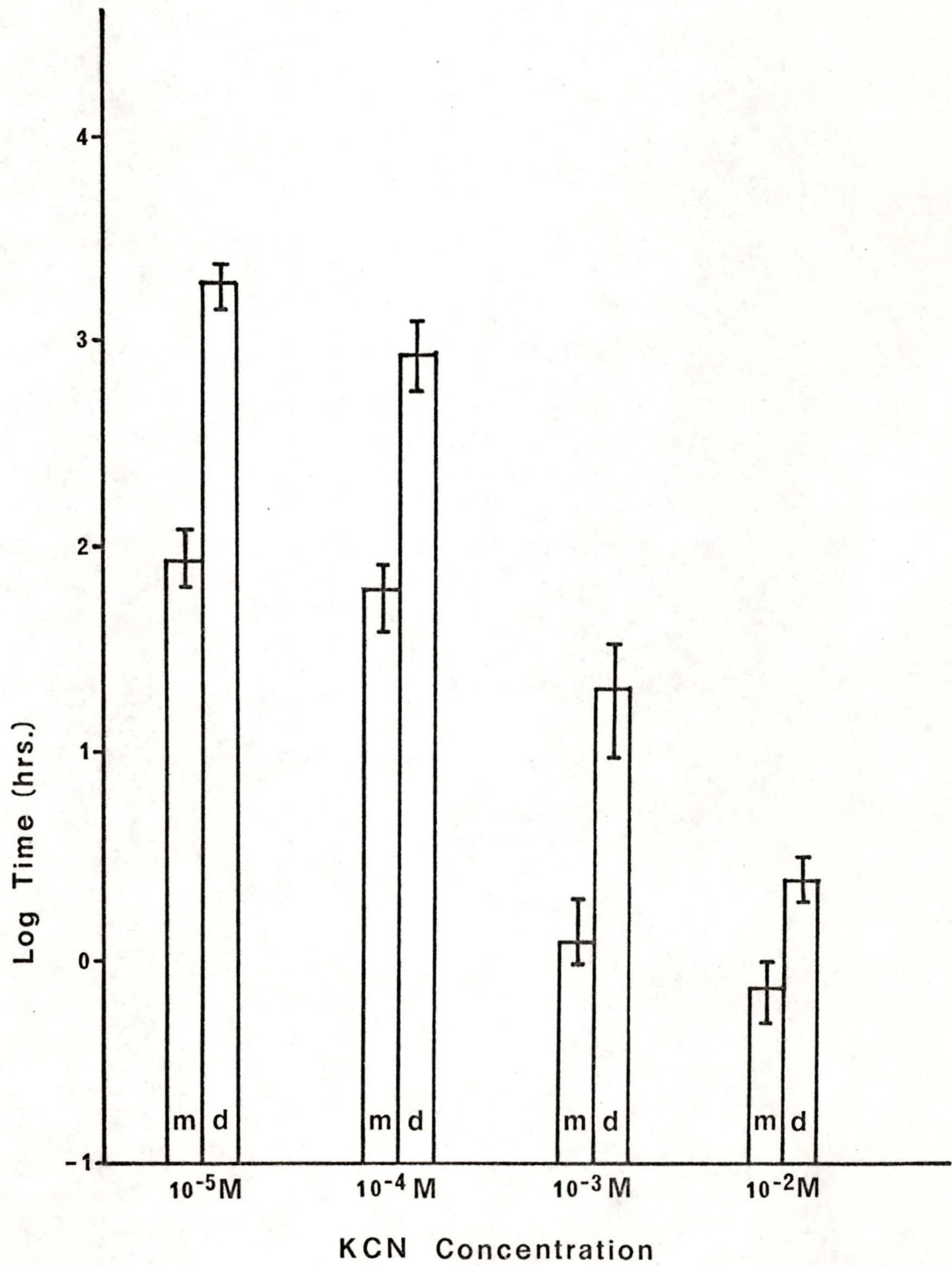
Control animals exposed to similar concentrations of KCl showed none of these responses in the times noted. The speed with which the $CN^-$ ion was able to enter the copepod in sufficient quantity to effect maxillary paralysis and death at the higher concentrations provides an indication of the efficiency with which the mechanism of the bulla and maxillary ducts is capable of conducting small dissolved ions from the fluid beyond the bulla (normally host tissue fluid in nature) to the haemolymph of the parasite.

(iii) Dye solutions

A number of dyes, when presented in solution as vital stains to live

Fig. 6 Effects of absorption of $CN^-$ via the bulla *in vitro* (capillary tube system): exposure time for appearance of response vs. KCN concentration. (Vertical bar = mean, vertical line = range; n = 20).

m - paralysis of second maxillae and cessation of pumping; d - death (cessation of all visible muscular activity).


4
3
2
1
0
-1
Log Time (hrs.)
m
d
m
d
m
d
m
d
10⁻⁵M
10⁻⁴ M
10⁻³ M
10⁻²M
KCN Concentration

adult female *S. californiensis*, provided direct visual evidence of both the considerable permeability of the bulla and maxillary duct walls and the general impermeability of the external body surface. These included three basic cationic dyes, neutral red, toluidine blue and methylene blue, with un-ionized molecular weights of 289, 306, and 320, respectively, and two acid anionic dyes, phenol red and uranin (the water soluble fluorescein disodium salt), both with un-ionized molecular weights of 376 (Gurr, 1965).

When presented via the bulla in saline solution each of these dyes readily penetrated the bulla and appeared within the lumen of the maxillary ducts. Rate of penetration varied considerably, depending primarily on the rate of maxillary contractions, but dye was generally visible in the maxillary ducts within 30 - 60 minutes of initial exposure. After a period of several hours the cationic dyes had usually penetrated the maxillary duct walls and surrounding glandular tissue and entered the haemolymph in sufficient quantities to cause staining of internal structures and colour the body fluids within the trunk haemocoel. Staining of internal structures decreased in intensity with increasing distance from the second maxillae, and increased in intensity with increased length of exposure time, until both internal organs and haemolymph became heavily stained. Internal staining first became apparent within the second maxillae, then anteriorly in the region of the mouth tube and suboesophageal ganglion, and finally within the haemocoel of the trunk, corroborating earlier observations based on movements of suspended haemocytes which suggested that most or all of the haemal fluid leaving the second maxillae flowed first anteriorly, then posteriorly into the trunk. Rate of penetration

of the dye varied widely between individual copepods in all experiments, some requiring considerably longer than others to acquire the same degree of colouration.

The anionic dyes penetrated the bulla only slightly more slowly than the cationic dyes in most instances, but required considerably more time than the latter to penetrate the walls of the maxillary ducts and enter the haemolymph in sufficient quantity to be detected under the microscope. Phenol red often did not become visible in the haemolymph for 24 hours, or longer, while uranin generally required several days, and even then frequently could not be detected with certainty. This much reduced rate of influx of anionic dyes with respect to that of the cationic dyes used could be related to charge differences, but is more likely due to the larger size and more bulky shape of the anionic dye molecules, with molecular shape probably being the more important factor. Neutral red, toluidine blue and methylene blue have almost identical, roughly linear structures, while uranin has a larger and much more bulky structure and phenol red occupies an intermediate position between these extremes (Appendix IV). A number of other dyes with even larger and more cumbersome molecular structures were also presented to the bulla in saline solution, including crystal violet (cationic, mol. wt. 408), janus green B (cationic, mol. wt. 483), light green SF yellowish (anionic, mol. wt. 793), and fast green FCF (anionic, mol. wt. 809) but none was able to penetrate beyond the lumen of the maxillary ducts.

It is therefore suggested in the absence of any evidence to the contrary that passage of dissolved materials into the copepod via the bulla and maxillary ducts is limited by the permeability of the duct walls and/or

surrounding glandular tissue, primarily on the basis of molecular size. The upper limit for molecular weight (ionized) appears from these experiments to be about 350, with probable additional limitations based on the physical shape or bulkiness of the ion and possible (though undemonstrated) limitations due to sign, magnitude and distribution of charges, and reactive subgroups. Regardless of whether active transport of some materials may occur across the tissue surrounding the maxillary duct, it is likely that a majority of the apparently wide range of substances capable of traversing this tissue do so in response to either passive diffusion processes or entrainment within intercellular spaces. If this is the case, it is entirely reasonable that molecular (or ion) size and shape should be predominant factors limiting this passage. However, it is not known at present whether the cuticle lining of the duct or the surrounding glandular tissue is responsible for this proposed molecular filtering effect.

It was also observed that none of the above-mentioned dyes was able to penetrate or even stain the external cuticle of the copepod, with the single exception of neutral red. This dye penetrated only at small points scattered over the cuticle, particularly on the ventral surface of the anterior trunk region. Staining at these points was localized and confined to small groups of cells beneath the thick cuticle, which were thought to represent dermal glands (as found in other copepods, including Lernaeopodidae), each opening to the surface of the cuticle via a minute duct or canal and pore. However, why only neutral red of the dyes tested should penetrate these structures is a mystery, particularly considering the very similar chemical structures and properties

of the three cationic dyes (Appendix IV). It is assumed for the moment that the fine canals are filled with a substance which for some reason permitted passage of only neutral red among the dyes tested, perhaps as a result of its slightly smaller molecular size (289, or 254 when ionized).

Dermal glands and pore canals of varied size and function are common in arthropods and have been recently described from the ventral surface of the "head" of another parasitic copepod, Pennella elegans Gnanamuthu, occurring partially embedded in the tissues of the flying fish, Cypsilurus sp. (Kannupandi, 1976). In this instance the pore canals were reported "... to contain proteins and carbohydrates", and the function of absorbing nutrients from the hosts tissue fluids was hypothesized. In this regard it is also interesting to note that in the free-living marine copepod Calanus plumchrus the dermal glands have been found to be involved in uptake of dissolved organic material (specifically glucose) from seawater (Chapman, 1975). Neither function seems likely in Salmincola, but there are a number of other possibilities, including for example a role in the transport of dissolved gases for respiration. Apart from the localized penetration of neutral red, the cuticle appears virtually impermeable to dissolved ionic substances, and unless the cuticle is permeable to water to some degree (which is possible but unlikely in view of its thickness, hydrophobic nature and lipid content), a bypass route for the passage of dissolved gases in aqueous solution would seem advantageous. These fine canals might also account for the results of preliminary volume regulation experiments (not included in this study) which indicate that the external body surface, though not necessarily the cuticle, is to some extent permeable to water molecules but impermeable to dissolved ions (Cousens, unpublished data). The surface area is admittedly minimal, but in the

absence of any external respiratory structures or areas of thin permeable cuticle which appear adapted for or even capable of respiratory exchange, the concept of "controlled access" respiratory surfaces may be feasible.

It was also noted that dye never appeared in the gut of copepods immersed in a dye solution, indicating that neither oral nor anal ingestion or "swallowing" of the surrounding medium occur in S. californiensis. Both of these have been observed in Artemia for the purpose of osmoregulation (Croghan, 1958b) and have been proposed for a variety of purposes including respiration in other crustaceans, when a more obvious explanation was not at hand (Kabata, personal communication). However, this would seem to be a most unwise and unlikely respiratory strategy for a freshwater organism, parasitic or otherwise, in view of the osmoregulatory problem of excess water influx that would likely result.

(iv) $^{14}C$-labelled metabolites

Exposure of the bulla of live adult female S. californiensis to either 1-$^{14}C$-glucose or a mixture of fifteen U-$^{14}C$-amino acids, in saline solution, for a period of three or more hours invariably resulted in a highly significant ($P<0.001$) increase in the $^{14}C$ content of the tissues and/or body fluids of the copepod (Table VI). A further highly significant ($P<0.01$) increase in $^{14}C$ content occurred with an increase in exposure to the $^{14}C$-amino acid mixture from three to six or more hours. Differences in $^{14}C$ content between the six-, nine-, and twelve-hour-exposure groups of the $^{14}C$-glucose uptake experiment were not significant, perhaps due in part to the combination of sample size (n=20) and the high degree of variability of uptake rates between individual copepods (probably as a result of variability in maxillary contraction rates). However, it seems

Table VI. Mean $^{14}C$ content of groups (n=20) of S. californiensis exposed via the bulla to saline solutions of (A) a mixture of fifteen U-$^{14}C$-amino acids or (B) 1-$^{14}C$-d-glucose. Means and 95% confidence limits are back-transformed from calculated logarithmic values (Appendix III). (** denotes statistical significance at the P<0.01 level.)

A Exposure to a mixture of fifteen U-$^{14}C$-amino acids in saline.

| Group (n=20) | Exposure time (hr.) | Mean cmp/mg. (antilog log cpm/mg.) | | 95% confidence limits $L_1$ | $L_2$ |
|---|---|---|---|---|---|
| 1 | 0 | 5.1 | ** | 2.97 | 8.73 |
| 2 | 3 | 152.5 | ** | 108.89 | 213.31 |
| 3 | 6 | 2140.9 | ] | 1534.32 | 2992.84 |
| 4 | 9 | 1708.3 | ** | 1088.46 | 2681.09 |
| 5 | 12 | 1882.8 | ] | 1087.22 | 3263.51 |

B Exposure to 1-$^{14}C$-d-glucose in saline.

| Group (n=20) | Exposure time (hr.) | Mean cmp/mg. (antilog log c /mg.) | | 95% confidence limits $L_1$ | $L_2$ |
|---|---|---|---|---|---|
| 1 | 0 | 6.7 | ** | 4.42 | 10.28 |
| 2 | 4 | 3670.2 | ] | 2490.33 | 5416.80 |
| 3 | 8 | 2828.4 | ** | 1581.62 | 5068.72 |
| 4 | 12 | 1988.2 | ] | 1345.32 | 2939.40 |

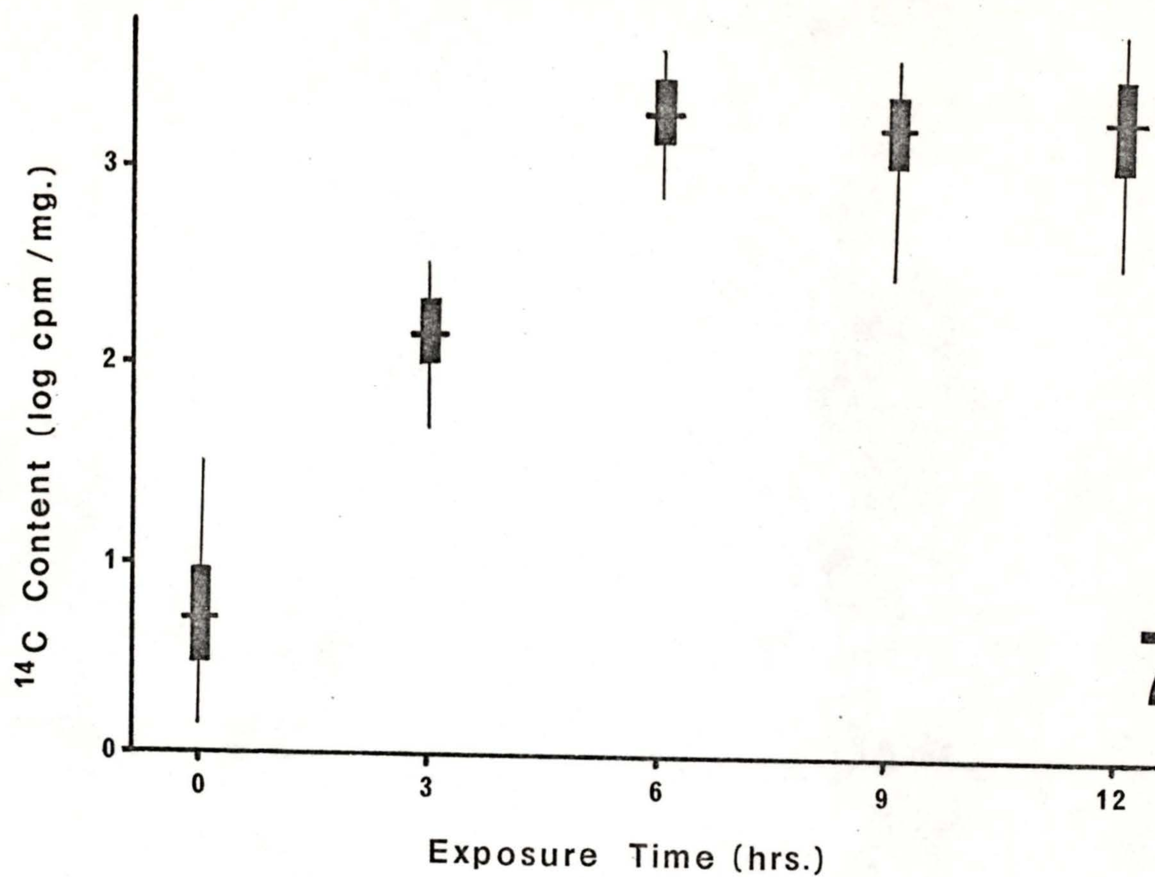
unlikely that this variability, due to the presence in each group of a few extremely low (by comparison) counts, is responsible for completely masking otherwise significant differences between the mean $^{14}C$ content for these groups, particularly considering the similarity between the upper range values for each group (Figs. 7,8). (If $^{14}C$ content were to continue to increase with exposure time in these groups, though this trend was not reflected in the group means due to biasing by a few extremely low values, the upper limit of the range would still be expected to increase with exposure time in a sample of 20 animals. This was clearly not the case in either experiment.)

The alternate explanation involves an initial rapid increase in $^{14}C$ content at the beginning of the experiment, followed by a levelling-off due to establishment of an equilibrium between internal and external concentrations of $^{14}C$-amino acids of $^{14}C$-glucose at some point in the system (Figs. 7,8). The nature of this equilibrium is unknown at present, and it cannot be determined from the present data whether the levelling-off of the uptake curves represents a dynamic equilibrium between internal and external concentrations across the maxillary duct system, or merely an artifact of the capillary tube system due to depletion of $^{14}C$-amino acid of $^{14}C$-glucose levels in the saline tube medium near the bulla during the initial uptake phase, and establishment of a stable concentration gradient in the stagnant tube medium at the lower end of the capillary tube. The latter is a distinct possibility which can only be discounted by development of a more elaborate method of bulla isolation involving some form of circulation of the tube medium or its equilavent.

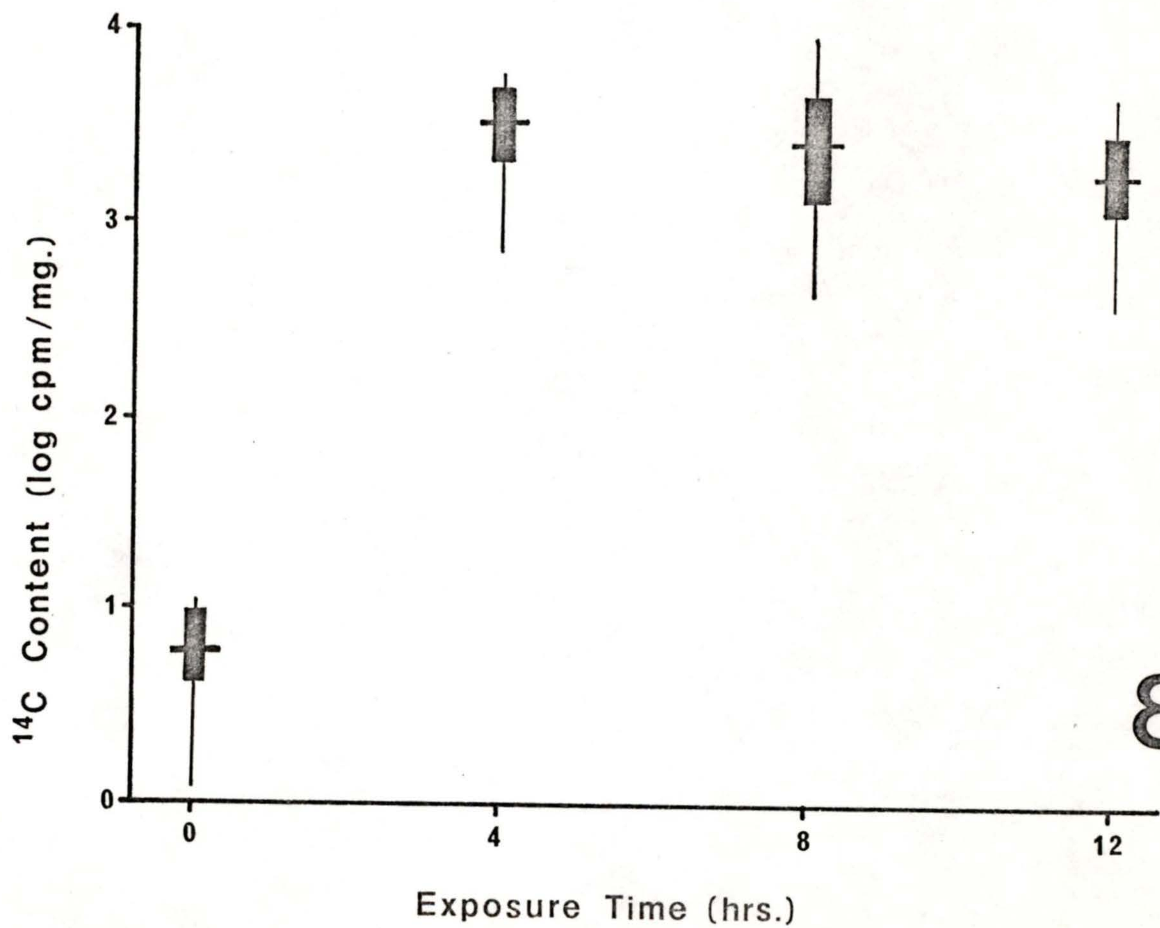
Nevertheless significant entry of both $^{14}C$-amino acids and $^{14}C$-glucose

Fig. 7 Absorption of U-$^{14}$C-amino acids via the bulla *in vitro* (capillary tube system): log $^{14}$C content (cpm/mg) vs. exposure time. (Horizontal line = mean, vertical bar = 95% confidence limits to mean, vertical line = range; n = 20).

Fig. 8 Absorption of 1-$^{14}$C-d-glucose via the bulla *in vitro* (capillary tube system): log $^{14}$C content (cpm/mg) vs. exposure time. (Horizontal line = mean, vertical bar = 95% confidence limits to mean, vertical line = range; n = 20).



$^{14}C$ Content (log cpm / mg.)
0
1
2
3
0
3
6
9
12
Exposure Time (hrs.)
7

$^{14}C$ Content (log cpm / mg.)
0
1
2
3
4
0
4
8
12
Exposure Time (hrs.)
8

via the bulla is clearly demonstrated, and it is interesting to note that the equilibrium level was reached considerably more quickly with $^{14}C$-glucose (< 4 hrs.) than with the $^{14}C$-amino acid mixture (5-6 hrs.). Whether this is a function of the relative concentrations of labelled compounds in the two media (1-$^{14}C$-glucose being present at 100 μM, while the averaging concentration of U-$^{14}C$-amino acids in the mixture was 2.5 μM and the total concentration was 38.2 μM) or the result of active uptake of $^{14}C$-glucose by the maxillary duct epithelium is a matter for speculation, on the basis of present data, as these experiments were not designed to yield information on relative uptake rates or methods of transport.

In a further experiment designed to demonstrate uptake of intact amino acids, extraction and separate $^{14}C$-analysis of free amino acid (i.e. 15% TCA-soluble) and protein-containing (i.e. 15% TCA-insoluble) fractions from four groups of five copepods each, after either 3 hr. or 12 hr. exposure to the $^{14}C$-amino acid solution, revealed the presence of considerable amounts of $^{14}C$ in both fractions (Table VII). Counts(cpm/mg.) were notably higher in both fractions for the groups exposed to the solution for 12 hrs. and in all groups some incorporation of $^{14}C$ into protein occurred. Percent of total $^{14}C$ in the protein fraction for each group was fairly constant regardless of exposure time, ranging from 8.8% to 15.2% with a mean of 12.3%. These results imply that the $^{14}C$-amino acids are being absorbed from solution intact and indicate that S. californiensis is indeed capable of obtaining amino acids via the bulla for incorporation into protein.

However, it is not possible to determine from these results which of the 15 labelled amino acids provided were taken up by the parasite. Nor

is it possible to determine from the presented data whether the observed uptake occurred by active or passive processes, as these preliminary experiments were desinged only to confirm or refute the hypothesis that uptake of dissolved amino acids and glucose via the bulla and maxillae was at least possible and could be demonstrated in vitro. (Demonstration of uptake in vivo on the host was not attempted, due to complications of metabolism of the labelled compounds by the host and the difficulty of separating uptake via the maxillae from uptake by feeding on host tissues containing $^{14}C$.) The rates of uptake presented are approximate due to the small number of animals tested in each group, and are therefore truly quantitative only in a comparative sense. In order to answer the more specific questions concerning uptake of metabolites from the host by this route, a far more extensive and detailed separate study would be required.

Table VII. Incorporation into protein of $^{14}$C-amino acids absorbed via the bulla by S. californiensis. Percentage values indicate partitioning of $^{14}$C (as cpm/mg dry wt of analyzed copepod tissue) between 15% TCA-soluble (amino acid-containing) and 15% TCA-insoluble (protein containing) fractions.

| Group (n=5) | Exposure time (hr.) | Protein-containing fraction cpm/mg dry wt | Protein-containing fraction % of total cpm/mg | Amino acid containing fraction cpm/mg dry wt | Amino acid containing fraction % of total cpm/mg |
|---|---|---|---|---|---|
| 1-1 | 0 | 2.10 | 33.3 | 4.21 | 66.7 |
| 1-2 | 0 | 2.27 | 33.9 | 4.42 | 66.1 |
| 2-1 | 3 | 101.04 | 8.8 | 1050.49 | 91.2 |
| 2-2 | 3 | 158.03 | 13.9 | 977.14 | 86.1 |
| 3-1 | 12 | 495.81 | 15.2 | 2761.36 | 84.8 |
| 3-2 | 12 | 540.46 | 11.4 | 4185.31 | 88.6 |

Uptake of glucose (mol. wt. 180) and at least some amino acids via the bulla and maxillary ducts is not entirely surprising in view of the permeability of this route to some vital stains having molecular weights of up to approx. 300, as noted in the previous section. However, the ability of Salmincola to obtain at least a portion of its nutritional requirements via the bulla and second maxillae has not previously been demonstrated or even seriously considered.

(v) Chemotherapeutic agents

The results of in vitro permeability tests with Ronnel, Trolene 20L (40% Ronnel emulsifiable concentrate), Ruelene, Ruelene 25E (25% Ruelene emulsifiable concentrate) and phenoxethol are summarized in Table VIII.

(a) Ronnel

Concentrations of up to 40 mg/l (=ppm) pure Ronnel dissolved in distilled water (i.e. an approximately saturated solution) failed to penetrate the external body surface of adult female Salmincola

in sufficient quantity and state of activity to produce observable indications of toxicity during an exposure period of 96 hours. Similar concentrations prepared in salmonid saline and presented via the bulla and maxillary ducts also failed to produce any observable effects over a 96 hour exposure period. It was thus concluded that pure Ronnel in aqueous solution was either incapable of entering by either route or entered so slowly that it was degraded and hence deactivated by the copepod before reaching toxic levels in the cholinergic junctions of the central nervous system. In either instance pure Ronnel shows no potential *in vitro* as a control agent for *Salmincola*.

(b) Trolene 20L (40% Ronnel emulsifiable concentrate)

Concentrations of 10, 25 and 50 µl/1 (corresponding to 4, 10 and 20 mg/1 pure Ronnel) failed to produce any indications of toxicity to adult female *S. californiensis in vitro* when presented either via the body surface or via the bulla and maxillary ducts, over an exposure period of 96 hours. (Higher concentrations of the emulsifiable concentrate could have been prepared and tested, but preliminary experiments shows such concentrations to be highly toxic to fish and hence impractical as an *in vivo* control method.) The emulsifiable concentrate formulation of Ronnel is therefore also considered to have no potential for use as a *Salmincola* control agent.

(c) Ruelene

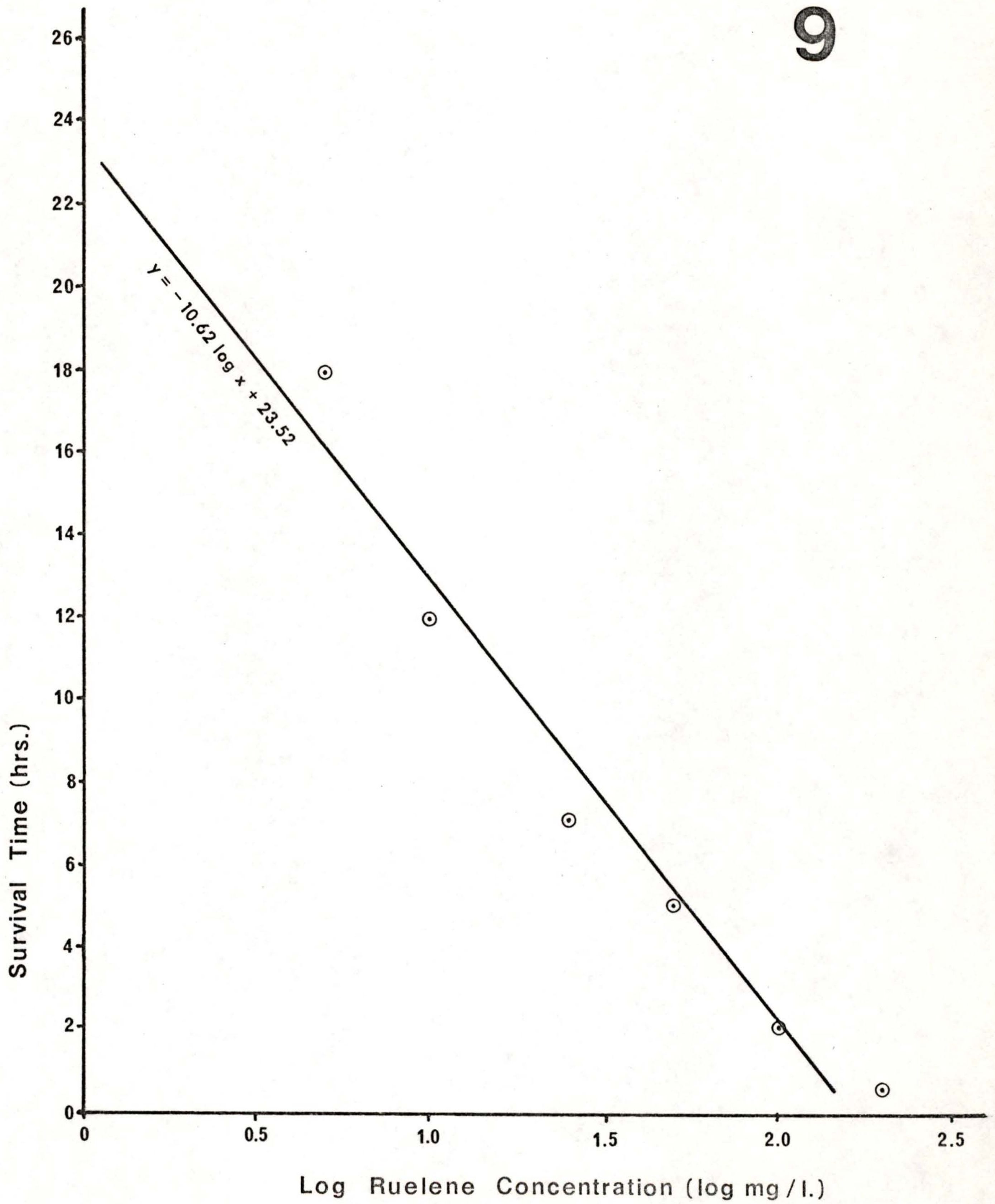
A wide range of concentrations of pure Ruelene proved toxic to *S. californiensis* when presented to the external body surface in

Table VIII. Effects of chemotherapeutic agents Ronnel, Ruelene and Phenoxethol on adult female S. californiensis, in vitro.

| Test compound and formuation | Concentration Emuls. conc. (ul/1.) | Pure agent | Penetration via external cuticle (n=20) +/- | Observed effects | Penetration via bulla and max ducts +/- | Observed effects |
|---|---|---|---|---|---|---|
| Ronnel: | | | | | | |
| Ronnel (98% pure) | | $10^{mg}/1$ | - | None (96 hrs.) | - | None (96 hrs.) |
| | | 20 | - | " " | - | " " |
| | | 40 | - | " " | - | " " |
| Trolene 20L (40% Ronnel) | 10 | 4 | - | " " | - | " " |
| | 25 | 10 | - | " " | - | " " |
| | 50 | 20 | - | " " | - | " " |
| Ruelene: | | | | | | |
| Ruelene (92+% pure) | | 5 | + | Lethal;18 hrs(12-26) | - | " " |
| | | 10 | + | " 12 ( 9-16) | - | " " |
| | | 25 | + | " 7 ( 5-10) | - | " " |
| | | 50 | + | " 5 ( 2- 8) | - | " " |
| | | 100 | + | " 2 ( 1- 4) | - | " " |
| | | 200 | + | " 0.5 (0.3-1) | - | " " |
| Ruelene 25E (25% Ruelene) | 10 | 2.5 | - | None (96 hrs.) | - | " " |
| | 25 | 6.25 | - | " " | - | " " |
| | 50 | 12.5 | - | " " | - | " " |
| Phenoxethol | | $1\ ^{ml}/1$ | + | anaesthesia 8 hr. (6-10 lethal -- | - | " " |
| | | 2.5 | + | anaesthesia 30-60 min. lethal 1-2 hr. | - | " " |
| | | 5 | + | anaesthesia 3-5 min. lethal 10-15 min. | - | " " |
| | | 10 | + | anaesthesia 1-2 min. lethal 5 min. | - | " " |
| | | 20 | + | anaesthesia 1-2 min. lethal 5 min. | - | " " |

distilled water. Lethal concentrations ranged from 5 $^{mg}/l$ (mean survival time 18 hrs., n=20) to 200 $^{mg}/l$ (mean survival time 0.5 hrs.), as indicated in Table VIII. Regression of mean survival times (Y axis) on the log of the Ruelene concentration (X axis) was highly significant ($P<0.001$), the calculated linear regression curve having the form $Y = -10.62 \log X + 23.52$, and accounting for 95.6% of the variation between mean survival times (stand. error of regression coefficient $m = \pm 1.14$) (Fig. 9). This indicates a linear decrease in mean survival time with proportionate increase in Ruelene concentration, as is typical of simple dose-related toxicity phenomena. However, while this realtionship can be considered to hold true over most of the range of concentrations tested, it cannot be accepted for either very high or very low Ruelene concentrations, at which points the true relationship must become asymptotic due to either cuticle penetration-rate limitation or the minimum mortality-producing threshold concentration. As examples of the former, the above regression line predicts instantaneous mortality at approx. 164 $^{mg}/l$ Ruelene (the X- intercept, i.e. exposure time = 0) and a mean survival time of -0.9 hrs. at 200 $^{mg}/l$ Ruelene, both of which are clearly impossible. Meanwhile, at the low-concentration end of the curve, the regression equation predicts a mean survival time of 23.5 hours at 1 $^{mg}/l$ Ruelene concentration (the Y-intercept, i.e. log X=0, X=1), while in fact mortality was not repeatedly observed at this concentration in a 96-hour exposure period. In other words, a threshold Ruelene concentration, below which copepods are not killed in a 96-hour exposure period, occurs somewhere between 5 $^{mg}/l$ and 1 $^{mg}/l$, perhaps due in part to the fairly rapid breakdown rate for this compound.

Fig. 9 Linear regression of mean survival time for *S. californiensis* on Ruelene concentration. (Standard error of regression coefficient $m = \pm 1.14$, $P < 0.001$; $n = 20$).


9
Survival Time (hrs.)
26
24
22
20
18
16
14
12
10
8
6
4
2
0
y = −10.62 log x + 23.52
0
0.5
1.0
1.5
2.0
2.5
Log Ruelene Concentration (log mg / l.)

Nevertheless, pure Ruelene is clearly capable of penetrating the cuticle of S. californiensis, either via the cuticle itself or via pore canals, with lethal results at concentrations of 5 $^{mg}/l$ (=ppm) or greater. Considering the general impermeability of the cuticle to most ions and molecules in solution, and the molecular weight of Ruelene (292), it is tentatively suggested that Ruelene penetration may occur in a manner similar to that observed for the vital stain neutral red (mol. wt. 289, ionic wt. 253.5). An alternative hypothesis might involve the partitioning of the weakly polar Ruelene molecule out of aqueous solution into the bound lipid component of the external cuticle, since this compound is far more soluble in lipid solvents than in water. However, any further evidence to support either of these hypotheses is presently lacking. The same degree of toxicity was not observed when copepods were exposed to similar initial Ruelene concentrations in vivo on infected sockeye smolts, though at the higher concentrations the fish were quickly killed (see Section IV: Chemotherapy).

When presented to copepods via the bulla and maxillary ducts in saline solution, all concentrations of Ruelene up to the approximate saturation level (200 $^{mg}/l$) failed to show any indications of toxicity. Since the bulla is freely permeable to considerably larger molecules in solution, it is probably safe to assume that Ruelene molecules were able to penetrate to the lumen of the maxillary ducts. However, the complete lack of the previously demonstrated toxic effect of this compound suggests that Ruelene molecules were unable to penetrate the duct walls and surrounding tissue in sufficient quantity during the 96-hour exposure period to induce these effects. It thus seems

unlikely that Ruelene could be absorbed from the host via this route *in vivo* in sufficient quantities to prove effective as a systemic control agent for *Salmincola*, and hence its value for this purpose would be only in relation to its ability to be taken up from peripheral tissues of the host in sufficient quantities to prove toxic during feeding activities of the parasite.

(d) Ruelene 25E (25% Ruelene emulsifiable concentrate)

Solutions containing either 10, 20 or 50 μl/l of Ruelene 25E (corresponding to 2.5, 6.25 and 12.5 $^{mg}/l$ pure Ruelene) in distilled water or saline failed to produce any indications of toxicity to *S. californiensis* adult females when presented either via the external cuticle or via the bulla and maxillary ducts for periods of 96 hours. The inability to penetrate via the maxillary ducts is consistent with previous results with the pure compound, but in view of the apparent permeability of the cuticle to pure Ruelene, the inability of the emusifiable concentrate formulation to penetrate via this route is curious. Two possible explanations for these seemingly conflicting results are that the emulsifying agent molecules, by associating with the Ruelene molecules, either increase the effective molecular size of the latter beyond that size which can pass through the permeable sites of the cuticle (assuming the "pore hypothesis" of penetration), or reduce the partition coefficient of the Ruelene molecules such that they pass from aqueous solution and into cuticle lipid less readily than similar molecules not complexed with emulsifying agents (assuming the alternate "cuticle lipid-partitioning hypothesis"). In either event Ruelene 25E would appear to be of little value as a control agent for *Salmincola*, since low concentrations cannot penetrate

the parasite, while higher concentrations are lethal to sockeye smolts.

(e) Phenoxethol

Phenoxethol concentrations of 2.5 $^{ml}/l$ or greater were toxic to S. californiensis when presented to the external cuticle in distilled water as a dip treatment. Concentrations below this level produced only sublethal anaesthesia which was reversible over a period of 12 - 24 hours by placing the copepod in distilled water without phenoxethol. At a concentration of 10 ml/l or higher the mean survival time was minimal and constant, anaesthesia occurring in 1 - 2 minutes and death occurring within 5 minutes. Consequently, a hyperbolic response curve results if these data are plotted, and not surprisingly a meaningful linear regression curve for concentration-dependent mortality cannot be fitted, as there are too few data points in the centre of the range of concentrations tested (e.g. 2.5 - 10 ml/l) where survival time might reasonably be expected to be a function of phenoxethol concentration alone. Elsewhere on the curve the survival time vs. concentration relationship is influenced by prolonged (up to at least 96 hr.) tolerance of low phenoxethol concentrations (e.g. below 2.5 ml/l) without mortality, or by the maximum rate of penetration of cuticle and/or minimum response time to this compound in high concentrations (e.g. 10 $^{ml}/l$ or greater), these effects causing both ends of the response curve to become asymptotic.

Though phenoxethol concentrations can be toxic to S. californiensis when presented as an external treatment, those concentrations found

to be capable of killing the parasite in a reasonably short period of time are one to two orders of magnitude greater than concentrations recommended for use as anti-fungal agents for fish (e.g. 0.1 - 0.2 $^{ml}/l$), and at least one order of magnitude greater than concentrations generally used for anaesthetizing salmonids (approx. 0.3 $^{ml}/l$). It therefore seems unlikely that phenoxethol dip treatments could provide effective Salmincola control without killing the host, since this compound appears to be more toxic to the host than the parasite. (This hypothesis was later confirmed, as indicated in the results of the preliminary chemotherapy attempts - Section IV.)

Presentation of similar concentrations of phenoxethol via the bulla and maxillary ducts in saline solution produced no indications of penetration into the haemolymph, with no resulting mortality or anaesthesia, over a period of 96 hours exposure. This apparent inability of the comparatively small phenoxethol molecule (mol. wt. 138) to penetrate via the second maxillary ducts is curious, considering the permeability of this route to much larger molecules (e.g. vital stains with molecular weights of circa 300 and glucose, mol. wt. 180). A satisfactory explanation is not available from present knowledge of the mechanisms of uptake of materials via the maxillary ducts. However, it is reasonable to suppose that processes of selection, perhaps in combination with processes of active or passive transport of substances of host origin may operate at the interface between the internal fluids of the parasite and the fluid contents of the maxillary ducts. If this is the case, the simplest explanation for the observed inability of phenoxethol (and also Ronnel and Ruelene)

molecules to cross this interface would be that such molecules, all of which are un-ionized, only slightly polar, and of low water solubility, fall into a class of substances which are somehow "screened out" and prevented from entering, perhaps on the basis of these or other common properties.

IV. Chemotherapy

(i) Dip treatments (Table IX)

The results of the external dip treatments indicate that none of the compounds tested appear capable of killing or even seriously affecting adult female _S. californiensis_ on sockeye smolts at concentrations up to and including those found to be rapidly lethal to the host fish, under the conditions of these experiments. This was to be expected for phenoxethol, since concentrations showing toxicity to _Salmincola in vitro_ (2.5 ml/l or greater) were well in excess of concentrations used to produce complete anaesthesia in salmonids (usually 1:3000, or 0.33 $^{ml}/l$). However, the relative toxicities of Ruelene to host and parasite were less obvious. _In vitro_ exposure of _Salmincola_ to a range of Ruelene concentrations suggested an approximate 24 hr - $LC_{50}$ somewhere between 5 and 10 $^{mg}/l$ for the copepod, while these results imply a similar 24 hr - $LC_{50}$ for 50 gm infected sockeye smolts. Thus some signs of toxicity to the copepod might be expected during the dip treatments, particularly in the 10 mg/l group where exposure for up to 24 hrs. extends beyond the mean survival time for _Salmincola_, found to be about 12 hours at this concentration. This was not the case, however, perhaps due to the rapid rate of degradation of Ruelene in aqueous solution and/or

Table IX. Results of dip-treatment chemotherapy with infected sockeye smolts (approx. 50 gm.)

| Test Compound | Emuls. conc. | Pure agent | Sockeye (O. nerka) smolts (n=10) mortality | sublethal effects | S. californiensis (20-30/fish) mortality | effects |
|---|---|---|---|---|---|---|
| Ruelene<br>(92+% pure) | - | 1 mg/1 | 0%/72 hr. | - occasional coughing | 0% | - none observed |
| | - | 2 | 0%/72 hr. | - coughing | 0 | " " |
| | - | 5 | 30%/48 hr.<br>90%/72 hr. | - coughing, distress,<br>loss of equilibrium | 0 | " " |
| | - | 10 | 70%/24 hr.<br>100%/48hr. | - coughing, distress,<br>loss of equilibrium | 0 | " " |
| | - | 15 | 60%/12 hr.<br>100%/24 hr. | - sever distress<br>erratic swimming<br>loss of equilibrium | 0 | " " |
| | - | 25 | 100%/ 4 hr. | - as above | 0 | " " |
| Ruelene 25E<br>(25% Ruelene) | 5 ml/1 | 1.25 | 0%/72 hr. | - coughing | 0 | " " |
| | 10 | 2.5 | 30%/72 hr. | - coughing, distress,<br>loss of equilibrium | 0 | " " |
| | 20 | 5 | 40%/24 hr.<br>100%/48 hr. | - sever distress<br>erratic swimming<br>loss of equilibrium | 0 | " " |
| | 50 | 12.5 | 100%/30 hr. | - as above | 0 | " " |
| | 100 | 25 | 100%/ 3 hr. | - as above | 0 | " " |
| Phenoxethol | - | 0.2 ml/1 | 0%/15min. | - anaesthesia in<br>5-10 min., occasional<br>coughing | 0 | " " |
| | - | 0.33 | 0%/ 5min. | - anaesthesia in<br>3-5 min., occasional<br>coughing | 0 | " " |
| | - | 1.0 | 0%/ 2min.<br>100%/ 5min. | - anaesthesia in<br>1-2 min., coughing,<br>distress | - | " " |
| | - | 2.0 | 0%/ 1min.<br>100%/ 2min. | - anaesthesia in 1 min.<br>severe distress | 0 | " " |
| | - | 5.0 | 100%/ 1min. | - fatal anaesthesia<br>within 1 min.; brief<br>severe distress | - | - partial anaesthesia<br>fully reversible<br>in 3-5 min. |

absorption and perhaps deactivation of Ruelene by some component of fish mucus. It is encouraging to note that the approximate 24 hr - $LC_{50}$ suggested in these experiments for sockeye smolts is in good agreement with the 24 hr - $LC_{50}$ values determined by accepted bioassay procedures for fry of several species of trout, which ranged from 26.2 ppm to 36.8 ppm of an approx. 25% Ruelene formulation, or from 6.5 mg/l to 9.2 mg/l pure Ruelene (Willford, 1967). Willford also noted little or no increase in toxicity (i.e. decrease in $LC_{50}$ concentration) for periods of exposure longer than 24 hours, up to 96 hours, which he attributed to rapid deactivation of Ruelene during the exposure period. This effect was counteracted by addition of fresh organophosphate at 24 hour intervals in the current study, and a noticeable increase in toxicity with increased length of exposure was observed.

(ii) Oral dosing

Three oral doses at 24 hour intervals of up to 10.0 mg each of pure Ruelene (approx. 200 mg/Kg body wt.), or up to 50 μl each of Ruelene 25E (approx. 12.5 mg/Kg body wt.), produced no apparent effect on either the fish or the attached adult female S. californiensis over a period of 120 hours. Regurgitation of the larger doses of either formulation was fairly common, though only fish which accepted and retained all three doses were included. Apart from regurgitation the fish showed no signs of distress or symptoms of organophosphate poisoning, and both host and parasite appeared normal at the end of the five day period. Similar results were observed with oral doses of phenoxethol ranging from 10 to 100 μl (0.2 to 2.0 ml/kg. body wt.),

though 50 µl/l was the largest dose accepted voluntarily, and larger doses were only retained if administered by force feeding to anaesthetized fish.

Both Ruelene and phenoxethol are considered to have systemic properties as chemotherapeutic agents and the latter is recommended for use in fish as a feed additive (Duijn, 1967), though their previously demonstrated inability to penetrate via the maxillary ducts would seem to limit their potential systemic activities against Salmincola to being taken up from peripheral host tissues in toxic quantities during feeding. This effect would presumably be most apparent in the region of the gills, where copepods actively feed on gill tissue and are seen to contain haemoglobin from the host's blood in their guts. Copepods attached elsewhere and feeding on more peripheral tissues not containing blood might be expected to encounter a lower level of a systemic control agent. In all cases copepods observed to contain fresh blood at the end of the five day period, indicating probable ingestion of blood during that period, were apparently healthy and active. This suggests that blood levels of the systemic compounds may not have reached toxic levels for Salmincola during the oral dosing period, at least not for a sufficiently long period of time to produce mortalities. Since the upper limits for oral doses in these tests were the maximum doses accepted by the fish without regurgitation, success with larger oral doses seems unlikely. In view of the rapid breakdown rate for Ruelene and the apparently rapid loss of phenoxethol from the blood and tissues of the host (as indicated by the rapid recovery of fish fully anaesthetized by a 15 min. dip in a 300 ppm solution,

usually within five minutes of being returned to the water) it also seems unlikely that more prolonged treatments with these compounds would be effective. Hence both Ruelene and phenoxethol would appear to be of no value as systemic control agents for Salmincola, though the systemic approach might well prove feasible with a more suitable compound (see Discussion section for further recommendations concerning systemic control).

(iii) Intraperitoneal injection of Ruelene

Intraperitoneal injection at 24-hour intervals with three identical doses of Ruelene in saline solution (200 mg/l), ranging in Ruelene content from 50 to 400 μgm per injection (from 1 to 8 mg/Kg. body wt./ 24 hr. period) failed to produce any observable response in either the fish or the attached copepods. Due to the low water solubility of Ruelene, 400 μgm was the maximum dose which could be administered by this method, as 2.0 ml. of the 200 mg/l Ruelene/saline solution was considered the maximum volume which could be safely injected into the body cavity of 50 gm sockeye smolts. As previously, copepods with freshly ingested blood in the gut at the end of the five day test period were active and apparently healthy, suggesting that in the host's blood toxic levels for Salmincola were not reached. Quite possibly larger doses could produce blood levels toxic to the copepod, if such levels could be tolerated by the fish, but a different solvent would be required as a vehicle for the dose, such as perhaps a combination of saline and dimethyl sulfoxide. It should be noted, however, that intraperitoneal injection is intended only as an empirical method for estimating the degree of breakdown of oral doses in the digestive tract of the fish (inconclusive here due to

dose limitations) and is clearly not practical as a general control method.

# DISCUSSION

The following section reviews the results of this study as they relate to three specific areas of interest concerning Salmincola; structure and function of the bulla and maxillary ducts, chemotherapy for infected fish stocks, and the evolutionary origin and specialization of the bulla and second maxillae as attachment structures, with resulting implications concerning the origin and development of the family Lenaeopodidae.

## I. Structure and Function of the Bulla and Maxillary Ducts

The origin, structure and function of the arrangement of the bulla and second maxillae unique to the family Lernaeopididae has long puzzled scientists interested in this group of parasitic copepods. Though the origin of the bulla as a secreted structure released from the frontal region was eventually explained (Friend, 1941; Kabata and Cousens, 1973), studies of its structure have been rare and generally for taxonomic purposes, while the internal structure of the second maxillae and maxillary duct have not previously been described in detail. This results in part from study of the marine members of the family, where the bulla is reduced, hardened and apparently specialized solely for the obvious function of attachment to the host, while the maxillary ducts and often the maxillae themselves are usually greatly reduced, sometimes almost beyond recognition.

Such is not the case in the freshwater branch of the family, characterized by Salmincola and its closely related allies. All members of this group possess well developed elongate second maxillae, a complete maxillary duct system and usually an unhardened, elastic and often comparatively large bulla. This system is quite clearly specialized for other functions beside simple attachment to the surface of the host, for which a more sturdy

elaboration of the larval attachment filament would seem to be quite sufficient.

The bulla of Salmincola has been shown to be composed of an inert non-living and highly permeable reticulum of interwoven fibers which permits free movement of dissolved substances by diffusion between its outer surfaces and the lumen of the unlined loop duct. This loop duct is merely a curved tubular space within the bulla matrix which is connected at each end to one of the maxillary ducts via a tubular insertion. Dissolved substances diffusing into the fluid within the loop-duct are actively pumped into the maxillary ducts by alternate contractions of the maxillae, at which point they are separated from modified epithelial tissues of the parasite by only a thin layer of overlying cuticle. The ducts serve as reservoirs for the fluid contained in this closed system, and the alternate maxillary contractions cause continuous movement of this fluid back and forth between one maxilla and the other, via the bulla. The fluid in this duct system may be regarded as an extension or dialysate of the tissue fluids of the host which continually bathe the bulla, and any dissolved material in that tissue fluid which is capable of diffusing through the bulla matrix and the thin permeable cuticle of the maxillary duct walls is potentially available to the parasite. Since the bulla has been demonstrated to be permeable to molecules and ions up to a molecular weight of at least 800, while dyes with ionic weights of up to to 350 have been observed to pass from the maxillary duct fluid to the haemolymph of the parasite, the range of host substances which might be absorbed via this route is quite considerable. Uptake of dissolved inorganic ions, glucose and at least some amino acids has been demonstrated in this study, though it is not yet known whether uptake of these materials involves active or passive transport mechanisms.

It has also been shown that the maxillary duct epithelium possesses the ultrastructural characteristics typical of a transporting epithelium combined with features suggesting an ability to synthesize intermediate metabolites such as lipids and proteins. Considered in combination with a demonstrated ability of the copepod to absorb relatively simple metabolites such as inorganic ions, amino acids and glucose via the bulla *in vitro*, and the arrangement of the transporting and synthesizing elements within the maxillary duct epithelium, it is logical to suspect that raw materials such as amino acids, low-molecular-weight sugars and fatty acids, in addition to ions, may be absorbed from the maxillary duct filtrate of host tissue fluids via the Type I cells surrounding the duct. These metabolites could then be utilized in the synthesis of more complex compounds within the Type II and Type III cells, which would then be released via the basal labyrinth and basement membrane into the haemolymph of the parasite. More complicated alternative hypotheses are also possible (though less likely in the author's opinion), such as synthesis and secretion of materials via the intercellular spaces into the maxillary duct and eventually into the host, in opposition to the demonstrated route of uptake of ions and metabolites, though this hypothesis would not well suit the ultrastructural details as described. A more thorough explanation of the processes involved is not possible from current knowledge of this system.

However, it is thus apparent that the relationship between *S. californiensis* and its host is far more intimate than previously believed due to the link via the bulla and maxillary ducts. Conversely, because of this close association and the resulting degree of access provided the copepod to its host's metabolite pools, the parasite is able to exist relatively independently of its surrounding environment. The most striking example of this is the copepod's apparent

complete lack of dependance on the dissolved ion content of the surrounding medium, since it is capable of satisfying its requirements for salt ions by direct uptake from the carefully regulated and comparatively infinite supply ever-present within the tissue fluids of its host. Hence the demonstrated ability of the copepod to exist for periods of up to three weeks in distilled water *in vitro*, as long as a supply of essential ions was present in the form of a saline solution in contact with the bulla. In fact the copepod not only seems to not require an external source of ions other than its host, but appears to have virtually eliminated movement of most ions across the external surface by providing a cuticle with a surface layer of bound lipid sufficient to render it hydrophobic. There appear to be minute passages or "pore canals" bypassing this lipid layer, perhaps for respiratory purposes, but movement of dissolved substances through these passages would seem to be under fairly strict control.

An understanding of the permeability and mechanisms of absorption of materials via the maxillary ducts, together with an appreciation of the relative impermeability of the external cuticle, is of profound interest and application in study of the biology of *Salmincola* and hence in the development of control and eradication methods for this troublesome parasite of salmonid fishes. It also appears than an understanding of the function of the bulla, second maxillae, and maxillary ducts as a system highly specialized for absorption of essential materials from the host may at last provide some long-sought-after clues to the oirgin of this primitive group of freshwater lernaeopodids, and hence to the origin of the family Lernaeopodidae.

## II. Chemotherapy and *Salmincola*

Despite a long history as a major pest of salmonid fish-hatching and culturing facilities in the northern hemisphere, *Salmincola* has stubbornly

resisted all attempts at its control to date (Kabata, 1970). In North America the only successful control method reported involved eradication of S. californiensis from the Mt. Shasta hatchery in California by repeated chlorination of the hatchery and entire headstream to kill all resident fish, followed by removal of dead fish and restocking of the hatchery (Gall et al, 1972). From the foregoing study of permeability in S. californiensis the reason for failure of control attempts less drastic than chlorination (and for success of the latter), is abundantly clear. The majority of chemicals traditionally used for parasite control in fish have been readily water-soluble ionic compounds applied as external dip treatments. Such compounds are generally incapable of penetrating the thick external cuticle, and enter the copepod only very slowly or not at all, thereby failing to reach toxic levels within the parasite's tissues. The more recently developed organic pesticides (e.g. organophosphates) which have been adapted to fish disease therapy may be able to penetrate the cuticle in some instances (e.g. pure Ruelene) and not in others (as with Ronnel and both emulsifiable fornumations), either as a result of molecular morphology or perhaps lipid solubility. However, these compounds are generally highly toxic to fish, and the lethal concentration for the host is usually exceeded before a lethal concentration for the copepod is reached (as observed in the dip treatments with pure Ruelene, though this compound is one of the less toxic organophosphates with fish). In the chlorination procedure all host fish are killed and removed from the system, thereby eliminating the parasite as well. This can be likened to using a hand grenade as a mouse-trap - thorough and effective, but not very selective and extremely untidy.

Many disease-control methods for fish are discovered largely by a process of random trial-and-error, with little or no regard for the biology

of the particular organism concerned, and this method is often adequate with many parasites which present no special problems such as inaccessability. Salmincola, by being impermeable to many substances is often essentially inaccessible, and hence some preliminary knowledge of its permeability to various types of compounds in solution is required before an efficient search for a control agent can proceed. With this preliminary knowledge, potential control agents can be selected for testing on the basis of whether they conform to a structural or chemical type likely to penetrate into the parasite, and then tested fairly rapidly and efficiently *in vitro* as described earlier. In this way only compounds proving toxic to Salmincola are tested for relative toxicity to the host, and complications such as mucus absorption and deactivation of the test compound can be detected.

Though the preliminary attempts to develop a control procedure in this study were unsuccessful, they provide a model for further experiments of this nature, and have yielded considerable information which might be of use in selecting potential control agents. There are three basic routes of approaching the matter of Salmincola control, and recommendations for each of these categories are outlined below.

(i) External dip treatments

The following characteristics may aid in penetration of the cuticle:

(a) Molecular weight should be as small as possible (e.g. 300 or less).

(b) Molecular morphology should be as nearly linear or straight-chainlike as possible, without bulky side groups.

(c) Low water solubility may be advantageous if penetration of cuticle involves partitioning from aqueous solution into cuticle lipid (emulsifying agents may be disadvantageous).

(d) Molecules without strong electrical charges (i.e. unionized, non-polar or only weakly polar) may penetrate most readily, although an ionized straight chain compound similar in structure to neutral red might also be effective.

In addition the compound must persist in the treatment solution long enough to penetrate the copepod in toxic quantities, without rapidly biodegrading or being absorbed and deactivated by fish mucus. It should also be remembered that organophosphates and carbamates must penetrate to the central nervous system of the copepod to be effective, while they will react with most components (i.e. all cholinergic junctions) of the nervous system in fish. Other compounds besides these should therefore be considered.

(ii) Systemic treatments

Though the initial attempts at systemic control of Salmincola were unsuccessful, these results should not be taken to imply that systemic control of this parasite is not possible, since these negative results could have been predicted in advance from prior knowledge of the inability of these compounds to penetrate via the maxillary route, and also their rapid breakdown rates and short term persistence in the host's tissues and body fluids. It should be noted here that a recent Norwegian study reports promising results in control of the ectoparasitic copepod Lepeoptheirus salmonis on

Atlantic salmon of about 2.5 kg. average weight, using oral application of the organophosphate Neguvon (Bayer), i.e. the chemical compound trichlorfon, as a feed additive at dose levels of about 80 ppm. (i.e. 80 mg/kg body weight, presumably) in seawater at 9-12$^{o}$C (Brandal and Egidius, 1977). In fact systemic control can be efficient and highly desirable, particularly in the form of feed additives, since the chemotherapeutic agent is directed toward its target organism, then degraded within the host, rather than being almost completely lost to the surrounding environment. With this fact in mind the following suggestions for selection of potential systemic control agents are proposed, in the hope that they may be of help in future work of this nature. It should be remembered that these suggestions apply to selection of a compound which could be administered to the host as a feed additive, and which could be absorbed from the host's tissue fluids via the second maxillae of the parasite.

(a) Molecular weight below about 350, preferably below 300.

(b) Linear or straight-chain molecular morphology (possibly including individual aromatic ring structures) but without bulky side groups or complex cyclic structures.

(c) At least moderately soluble in water.

(d) Compound should dissociate readily into ions in aqueous solution, or if not ionic should exhibit appreciable polarity.

(e) Compound should not produce initial paralysis of the second maxillae, since without continued operation of the maxillary pump further absorption would be greatly reduced and lethal levels in the parasite might not be achieved.

In addition, the control agent should have a wide safety margin between host and parasite toxicity levels, since voluntary feeding and hence individual dose levels would be difficult to control, and also should obviously persist long enough and at sufficiently high levels between feedings to prove toxic to the parasite but not the host. Finally, residue retention times would have to be determined and Food and Drug requirements met before the compound could be approved for use on fish destined for human consumption.

Potential systemic control compounds should first be tested to determine maxillary penetration and toxicity to Salmincola in vitro, using the described capillary tube technique or an equivalent method for isolation of the bulla, so that even compounds proving unsuccessful will contribute to a better understanding of maxillary penetration requirements. Any compounds proving effective should then be tested on infected fish by a controlled dose method, such as the gelatin capsule technique, to determine toxic levels and hence the safety factor before general feed-addition is attempted.

Other more general approaches to systemic control of Salmincola are possible, such as application by immersion dip treatments rather than feed addition, with absorption of the control agent into the fish from the surrounding water, or the use of materials which need not be absorbed via the maxillae and could be ingested by the copepod during feeding on host tissues. It should be kept in mind, however, that Salmincola appears to restrict its feeding activities almost entirely to the epithelial tissues of its host, with some ingestion of blood on the gills (Kabata and Cousens, 1977), and hence it is unlikely that systemics requiring ingestion by the copepod would reach toxic levels in the epithelial tissues of the host (other than perhaps on the gills) without external application by dip treatments. The use of a chemical which could be absorbed and retained but not deactivated

by host mucus prior to ingestion by the copepod is an interesting possibility which should at least be considered. The limitations of these methods would be somewhat different from those outlined for oral systemics, but not necessarily less restrictive or fewer in number.

(iii) Metabolic control methods

It is apparent from the foregoing recommendations and experimental results that the major problem in development of control measures for Salmincola, or indeed for most difficult-to-control parasites of fish and other organisms, is the finding of a compound and application procedure which provide an adequate safety factor between toxicity to the parasite and to the host. This is particularly true of compounds which inflict damage by the same mechanism in both host and parasite, such as organophosphate and carbamate pesticides which operate by deactivation and inhibition of acetylcholinesterase at cholinergic nerve junctions. Such compounds can only be effective in controlling a particular parasite if the relative toxicities of that compound differ fairly widely, these depending upon relative blood and tissue volumes and relative susceptabilities (i.e. grams-absorbed-per-kilogram-body-weight tolerated).

One method of avoiding these complications is to develop control measures which specifically disrupt some metabolic process in the parasite without interfering severely with the metabolism of the host. A particularly good example concerns a method proposed for metabolic control of cattle grubs by manipulation of levels of amino acids in the hosts tissue fluids, such that amino acids required by the parasite could be made unavailable for a period of time (Kasting and McGinnis, 1966). The technique first involves determination of required amino acids in relation to those which can be

synthesized by the parasite, by injection of U-$^{14}$-d-glucose, incubation, and subsequent extraction, separation and identification of amino acids from the parasite. Those which can be synthesized will contain $^{14}C$, while those required from the host will be unlabeled. This technique should be readily adaptable to *Salmincola in vitro*, since the copepod has already been shown to absorb $^{14}C$-glucose from saline solution via the bulla and second maxillae. If the amino acid requirements of the copepod should prove sufficiently different from those of the host fish, introduction to the host-parasite system of chemical agents to temporarily block or disrupt synthesis or metabolism of specific amino acids might seem justified.

This is but one example of a number of possible metabolic control processes which might be attempted, once sufficient biology and biochemistry of the host-parasite system is understood to permit exploitation of comparatively minor but distinct differences in metabolism. However, such rewards are the result of extensive and sophisticated research, well beyond the level of random trial of potentially toxic pesticides by conventional methods. Without such research it may never be possible to salvage valuable salmonid stocks which have become infected with *Salmincola*, and which are consequently destined to destruction in freshwater fish culture facilities at present.

III Evolutionary Origin of the Bulla and Maxillary Duct System

The evolutionary origins of the bulla and the unique mode of permanent attachment by the bulla and second maxillae have long been a puzzle to those interested in the Lernaeopodidae, but lack of information concerning any other functions that this system might perform, other than the obvious function of attachment, has until now prevented any logical speculation about these origins. Study of the bulla has been almost entirely descriptive in nature and has been confined largely to marine forms in which the bulla and maxillae show secondary reduction and specialization strictly for attachment, while the absorbtive function appears to have been abandoned. In fact the developmental origin of the bulla as a secretion product from a gland in the frontal region of the chalimus IV female was described only recently (Friend, 1974; Kabata and Cousens, 1973), from life cycle studies of two members of the more primitive freshwater branch of the family (Salmincola salmoneus and S. californiensis). In addition, much of the earlier work was fraught with misconceptions about life cycles and confounded by the formerly confused state of the taxonomy in this group. It is therefore not entirely surprising that the origin and functions of this novel attachment system have remained unknown.

It is known that the primitive freshwater branch of family Lernaeopodidae, and the Salmincola group in particular, considerably pre-date their existing marine relatives (Kabata, 1977), and this group thus appears to be a likely candidate for study in search of clues to the original functions and advantages of the bulla and second maxillae, and perhaps to the evolutionary origin and development of this complicated mechanism of attachment. In this group alone the bulla, second maxillae and maxillary ducts are present in the fully

developed and functional form. Unfortunately, earlier stages in the evolution of these structures do not exist today and cannot be determined from fossil records, so the sequence of evolution cannot be deduced from examination of a series of organisms representing lesser degrees of adaptation, but can only be hypothesized from observation of the end products of this sequence. The following is an attempt based on currect knowledge, including the results of this study, to reconstruct in logical sequence a series of steps which might have resulted in development of the bulla and maxillary duct system as found in primitive fresh water lernaeopodids today, with consequent implications concerning a possible origin for family Lernaeopodidae in association with a fresh water habitat. This sequence is presented in brief diagrammatic form in Fig. 10.

Starting with the generalized podoplean ancestor of order Siphonostomatoida (Kabata, 1977) only a few basic assumptions need be made, i.e. possession of the primitive Caligus-type mandible diagnostic of Siphonostomatoida, a typical ten-stage life cycle as still found in Caligidae (e.g. two nauplii, one copepodid, six "post-copepodid" stages, and the reproductive adult) and a free-living or perhaps micropredatory habit, probably in a marine environment. The first step in the sequence involves the adoption of a parasitic mode of existence in association with a former prey organism (perhaps an invertebrate or archaic ancestor of the earliest fishes) during the copepodid stage of the life cycle, with subsequent modification of the main grasping appendages of the copepodid (the second antennae, second maxillae and maxillipeds) into hook-like structures for more secure attachment to the host. However, once an obligate ectoparasitic association is formed, and even more so as the free-swimming and host-locating abilities of later larval stages are lost during

Fig. 10 Proposed sequence of events in evolutionary development leading to the present-day Lernaeopodidae.

Family Lernaeopodidae

other Lernaeopodiformes

Caligiformes

LERNAEOPODIFORMES

-subsequent loss of uptake function in several lines of family which recolonized the marine habitat

-modification of bulla and maxillary duct system for ion and nutrient absorption from host in fresh $H_2O$

-origin of prototype bulla as anchoring device for adult female, perhaps on armoured bony fish

-suppression of adult and second postchalimus stages by development of neotenous first postchalimus

-permanent association of adult female copepod with a single host individual; adult sessile

-transfer of filament base from rostral region to tips of second maxillae between moults

-association of second maxillae with frontal filament, perhaps for its manipulation or grasping at moult

-suppression of both free-swimming nauplius larval stages; passed in egg prior to hatching

-(appearance of multiseriate egg-sacs; advantages uncertain at present)

-invasion from marine to fresh water environment (perhaps on primative armoured fishes?)

-adoption of frontal filament by all four chalimus larval stages

-appearance of permanently attached post-copepodid stage and development of larval frontal filament

-loss of larval free-swimming and host-locating abilities

-development of hook-like grasping appendages (second antenna, second maxilla, maxilliped )

-adoption of parasitism during copepodid stage

-assume: Caligus - type mandible (simple blade)
ten-stage life cycle
free-living or micropredatory habit
probably marine

GENERALIZED PODOPLEAN ANCESTOR

adaptation to the host, any crustacean larva is faced with the major problem of maintaining attachment to its host during subsequent moulting periods. Hence it would be advantageous to possess some form of attachment to the host which is secure but which leaves the appendages free to shed their outer cuticle layer during each successive moult, these requirements being met in this instance by development of the larval frontal filament and button as found in all known Siphonostomatid larvae (Kabata, 1970). This unique form of attachment consists of a small plug or button buried in host epidermal tissue and often cemented to a supporting structure (such as a scale, fin ray or gill ray), and a long narrow filament with an enlarged basal portion firmly attached to the frontal region of the larva. It is extruded from the frontal region of the copepodid during initial attachment (Kabata and Cousens, 1973), and might possibly have arisen by modification of the secretion product of an anterior cement gland, of the type found in settling barnacle nauplii. The frontal filament appears to have proven highly successful, acting as a "safety line" to ensure secure attachment to the host during moulting, and has become a basic diagnostic feature of siphonostomatid larvae. Eventually this form of attachment was adopted by at least the first four post-copepodid or chalimus stages, the filament base being re-cemented to the frontal region by a fresh drop of secretion from the frontal gland at each moult. This type of life cycle, with two free-swimming nauplii and a free-swimming infective copepodid, four chalimus stages attached by the filament, two unattached post-chalimus or preadult stages and unattached freely-mobile adults of both sexes is typical of existing members of family Caligidae (Kabata, personal communication).

At this point a major division occurs in the siphonostomatid ancestral stock, one branch, represented by sub-order "Caligiformes" retaining this basic

plan of life cycle, and the other branch, represented by sub-order "Lernaeopodiformes", undergoing a series of major modifications. It is suggested in the absence of any evidence to the contrary that this period of major modification of the basic life cycle may have coincided with transfer from a marine to fresh-water environment, these modifications resulting at least in part from new selective pressures encountered in this new environment. The host or series of hosts involved during this transition period will likely never be known with certainty, but it is tempting to suppose that they may have been the first fish-like colonizers of fresh water, presumably the ancestors of cartilagenous and bony fish.

A major change in the life cycle of the proto-lernaeopodid ancestor which may have occurred at this time as an adaptation to parasitism on primitive fish in a fresh water environment involves suppression of both free-swimming naupliar stages. In existing lernaeopodids these are passed in the egg, which hatches coincident with the moult from the second nauplius to the infective copepodid (Kabata and Cousens, 1973), thereby eliminating all non-infective free-living larval stages. (The development of multiseriate rather than uniseriate egg sacs is another modification which may have occurred at about this time and is now typical of existing lernaeopodids, though the adaptive value of this feature is uncertain at present.)

At this point in the evolutionary sequence a new feature is introduced in the attached chalimus larval stages - transfer of point of attachment of the filament base from the rostral region of the chalimus larval stages to the tips of the second maxillae. It is impossible to determine with certainty whether this transfer occurred before, after or coincident with

adoption of the filament by all the chalimus stages, but the lack of evidence of this modification having ever occurred in the Caligiformes, in which rostral attachment of the chalimus stages to the filament has been retained (e.g. Caligus), suggests that use of the second maxillae for this purpose appeared considerably later,after separation of the ancestral stocks. However, within the Caligiformes there is considerable use of the second maxillae of chalimus stages for tending and manipulating the filament, and possibly for gripping the filament base during the moulting process, such that the second maxillae of chalimus larvae have become extensively modified for this purpose in the Caligidae (Kabata - personal communication). The permanent association of the second maxillae with the filament base as found in the Lernaeopodiformes may thus be seen as a logical extension of this process, providing more secure attachment of the chalimus stages during the moulting process. Prior to each moult the filament base is transferred back to the rostral region by the second maxillae, a new drop of secretion is added from the frontal gland, and the barbed tips of the new post-moult maxillae are withdrawn from their cuticular sheaths and embedded in this secretion before hardening occurs. The entire filament reattachment process is thus completed within the old cuticle (which is still firmly anchored to the filament by the shed cuticle of the pre-moult second maxillae), after which the moult proceeds and the surrounding "bag-like" exuvium eventually splits and is finally cut away by the mandibles. The entire process was described in detail for Salmincola by Kabata and Cousens, (1973)and is thought to be basic to lernaeopodid ancestral stock.

With permanent commitment of the second maxillae to the function of attachment to the host during the chalimus larval stages, and consequent

release of the rostral region and frontal gland from this function on a full-time basis, the stage is set for development of a bulla. The sequence of development of this structure remains uncertain at present, since the status of the preadult stages at this point in the proposed evolutionary sequence is unknown. However, it can be presumed that the advent of the bulla or its immediate precursor was preceded by a tendency for permanent association with a single host individual during both chalimus and post-chalimus stages of the life cycle. In this event the supposed function of the pre-adult stages as free-swimming dispersal stages for location of the final host becomes superfluous as the adult becomes an obligate parasite on the larval host. In fact, with the preadult stages being attached to the host only by the second antennae, maxillipeds and possibly prehensile second maxillae (i.e. no filament as a safety line), the subsequent two moults become distinctly disadvantageous and have been eliminated in existing lernaeopodids, presumably by condensation of the latter two stages within the first preadult. This could be accomplished by a process of neoteny, i.e. acquisition of sexual maturity and reproductive capability by a preadult larval stage.

With elimination of the post-chalimus moults, premanent anchoring of the first preadult (now the reproductive adult) female becomes feasible and advantageous in terms of increased security of attachment to the host, the second maxillae already being to some extent modified for this purpose by way of their function in anchoring of chalimus stages to the filament. (The neotenous larval male must obviously remain mobile but is confined to a single host individual.) The result appears to have been a prototype bulla, produced from the frontal gland of female chalimus IV larvae and probably

resembling the larval button and filament base, without the intervening long filament. This may have come about in the same manner as proposed for origination of the larval filament, the frontal gland initially producing a cement-like substance in which the tips of the second maxillae could be embedded and perhaps securely glued to a solid sub-epithelial structure. In this connection it should be remembered that primitive fish of this period in fresh water were covered almost entirely by bony plates, a factor which may have had a profound influence on the development of the Lernaeopodidae.

At this early stage of development the site of adult attachment on the host was likely a function of larval settlement site, which in existing lernaeopodids is largely determined by where the infective copepodid happens to first contact its host. If this were the case the adult would have to be capable of attaching and surviving at almost any point on the surface of a fish. On the heavily armoured bony fish of this time the adult would therefore likely need to be able to attach to a thin epithelial layer overlying bony plates, and to survive on a diet of epithelial tissue and mucus. A similar situation can be seen today in the fresh water lernaeopodid *Tracheliastes* which cements its atypical bulla to the surface of a scale, possibly reminiscent of the primitive condition. Preference for adult attachment sites differing from those of larval settlement, as seen today in *Salmincola* (Kabata and Cousens, 1977), may have arisen later in the evolutionary sequence, as a result of further adaptation to the host and the selective pressures of an ectoparasitic existence. These pressures might include competition for space on the host, increased site specificity reducing competition for space at a particular site and eventually resulting in formation of new species. Since bulla morphology in existing Lernaeopodidae is very much related to attachment

site and particularly the nature of the host tissue in which the bulla is implanted (Kabata and Cousens, 1972, 1977), considerable further adaptation of the attachment structure or prototype bulla would be expected. The argument for development of adult attachment site specificity after permanent adult attachment by the prototype bulla is supported by the fact that mobility and attachment site selection by the larval female is accomplished not by a post-chalimus stage, as might be expected if site specificity arose prior to permanent adult attachment, but rather by the greatly prolonged chalimus IV stage after breaking away from the filament (a risky process with a high rate of failure and mortality), while the chalimus IV male remains with the filament until the next moult to the adult (Kabata and Cousens, 1973).

It is reasonable to suppose that the original prototype bulla in its simplest form probably consisted of a small sub-spherical or flattened disc-like structure similar to the larval button, quite possibly cemented to a solid sub-epithelial support (e.g. a scale or bony plate) and serving to anchor the tips of the second maxillae to the host. Adaptation to host, attachment site and surrounding tissue type might be expected to produce considerable readiation from this primitive condition, but such does not appear to be the case from examination of the existing fresh water lernaeopodids. All have a bulla conforming to a common plan with the single exception of the _Tracheliastes_-type bulla mentioned previously (Kabata and Cousens, 1972), and it is suggested that the latter represents an intermediate condition between the primitive prototype bulla and the typical fresh water-lernaeopodid-type bulla as found in _Salmincola_. (This is not to imply that _Tracheliastes_ itself is necessarily intermediate between primitive and more recent forms; only that it has retained or reverted to a primitive condition of the bulla

and method of attachment in association with its primitive heavily armoured host, the sturgeon.) The bulla of Tracheliastes is a simple conical affair with its subanchoral surface cemented to a scale, and could be derived from the hypothetical prototype bulla by simple enlargement due to incorporation of fluid-filled spaces which become inflated after bulla implantation and attachment. Inflation is a common feature of fresh water lernaeopodid bulla implantation and is a logical means of increasing the surface area and anchoring ability of the bulla after implantation, since this structure must necessarily be of minimal size when initially produced due to the small size of the chalimus IV larva. This is particularly true once the cementing of the bulla to a scale is abandoned and replaced by implantation into softer tissues, as in the remaining members of the fresh water branch of the family.

With implantation of the bulla in soft tissues and its inflation with fluid, perhaps as a result of a combination of increased attachment site specificity and the general reduction in bony armour of primitive fresh water fishes, development of the absorptive function is but a series of small steps. In fresh water an organism is continually faced with the task of obtaining and retaining sufficient ions, and any modification which increases the availability of ions is likely to be strongly favoured by selection processes. The fluid filled spaces within the bulla would acquire ions from the surrounding tissue fluids of the host by simple diffusion through the permeable bulla matrix, and following bulla implantation the attached copepod would be presented with an abundant supply of essential ions of host origin, via the ducts and tissues responsible for production of the fluid contents of the bulla. Modification of the secretory ducts and epithelium for ion uptake, connection of the spaces within the bulla to form a loop duct and development of the maxillary pump system for increased efficiency of transfer are useful

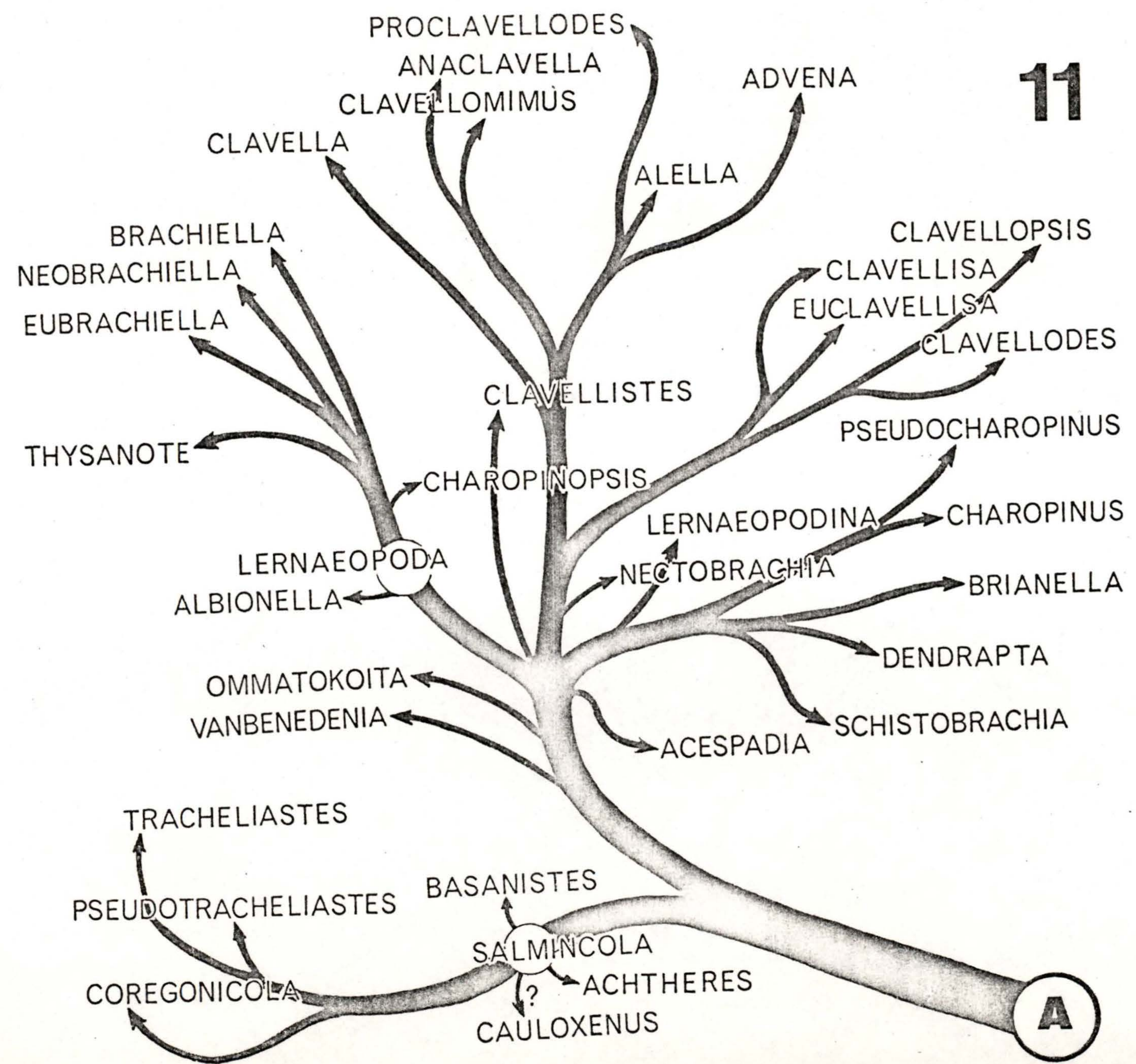
and relatively minor steps, once contact with the internal environment of the host via the bulla has been achieved.

Development of this unique method of acquiring essential materials from the host without causing continuous severe damage to the surface tissues likely ensured the success of the lernaeopodids as parasites of fresh water fishes, since the necessary materials could now be obtained with little risk of secondary infection from fungus or bacteria which might otherwise upset the host-parasite relationship prematurely. Success as a parasite requires at least the limited success of the host, such that the parasite survives to reproduce. Consequently, development of means of survival and nutrition which minimize the parasite-induced insults to the fish is advantageous, in order that the copepod generates no more damage than can be tolerated by the host over a period of time adequate to accommodate the life cycle of the parasite. As such, the ability of lernaeopodids to gain access to the internal environment of their host with minimal prolonged surface damage is paralleled in a number of other groups of parasitic copepods (e.g. Lernaeoceridae, Lernaeidae, and Sphyriidae), but in every other case the access has been gained by burrowing and penetration of the oral region into the host's tissues, tissue fragments and/or fluids being ingested directly (Kabata, 1970). The lernaeopodid approach is thus unique and one is tempted to search for a reason to justify this uniqueness. Apart from the contributing fact that the second maxillae are committed to an attachment function during the chalimus larval stages, the most obvious and logical reason seems to be that attachment by burrowing into the host's tissues may not have been possible or feasible during the earlier stages of the development of parasitism in this group, as would have been the case if

these early stages had occurred on the first heavily armoured ancestors of fishes in fresh water during the upper Silurian or lower Devonian eras. The necessary site specificity for location of soft tissues for burrowing may not have been developed until much later, after the basic method of attachment to bony armour by bulla and second maxillae had become fixed.

It is generally accepted that the cartilagenous fishes developed in fresh water but became almost entirely marine during the upper Devonian and Permian, while the ancestors of the bony fishes remained primarily in fresh water until Triassic and Jurassic times. Considerable evidence has now accumulated to suggest that lernaeopodids arose as parasites of the cartilagenous fishes prior to their major marine colonization, at which time these copepods became marine along with their hosts to give rise to the present day lernaeopodid parasites of sharks, skates and rays. In fact, the most primitive existing marine lernaeopodid, Vanbenedenia, closely resembles Salmincola in many respects and occurs only on holocephalans (e.g. the rat fish Chimaera), both being archaic survivors of that first marine colonization by cartilagenous fishes. The existing branch of the family characterized by Salmincola and its relatives has retained its ancestral ties with fresh water by developing an association with the freshwater phase of anadromous fishes and with related forms which have adopted a permanent fresh water existence. Consequently, primitive ancestral structures and conditions have been retained, including the structure and function of the bulla, second maxilla and maxillary duct as herein previously described. During the processes of adaptation of evolving groups of fishes from fresh water to marine environments and vice versa, which presumably could have occurred on a number of occasions, the ancestral fresh water lernaeopodid

Fig. 11 Taxonomic relationships within family Lernaeopodidae (from Kabata, 1977).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PROCLAVELLODES
ANACLAVELLA
CLAVELLOMIMUS
ADVENA
CLAVELLA
ALELLA
BRACHIELLA
CLAVELLOPSIS
NEOBRACHIELLA
CLAVELLISA
EUCLAVELLISA
EUBRACHIELLA
CLAVELLODES
CLAVELLISTES
PSEUDOCHAROPINUS
THYSANOTE
CHAROPINOPSIS
LERNAEOPODINA
CHAROPINUS
LERNAEOPODA
NECTOBRACHIA
ALBIONELLA
BRIANELLA
DENDRAPTA
OMMATOKOITA
VANBENEDENIA
SCHISTOBRACHIA
ACESPADIA
TRACHELIASTES
BASANISTES
PSEUDOTRACHELIASTES
SALMINCOLA
COREGONICOLA
?
ACHTHERES
CAULOXENUS
A

stocks may have served as a reservoir for colonization of new fresh water stocks and later reinvasion of the oceans, which also may have occurred more than once.

The marine lernaeopodid parasites of teleost fishes are considerably more advanced than both their relatives on cartilagenous fishes and the even more primitive fresh water members of the family, as indicated by such morphological characters as degree of modification of the mandible from its primitive simple condition and general morphology of the abbreviated male (Kabata; personal communication). It should be noted that an alternate hypothesis proposing derivation of the Salmincola-type bulla and maxillary duct structure and function from structures currently considered to be vestigial remnants of this system in existing marine lernaeopodids therefore implies an evolutionary trend throughout the family operating in direct opposition to the sequence of development of these groups as proposed by Kabata (Fig. 11, from Kabata, 1977).

In addition, further extensive modifications of the life cycle and the morphology of the male in one marine group (such that all four chalimus stages are reduced to a single "pupal" stage and the resulting sequence of ontogeny consists of only egg, copepodid, "pupa" and adult stages) set this group far apart from all other marine lernaeopodids as at least a separate sub-family, the Clavellinae (Kabata; personal communication). The lack of any recorded intermediate stages between this latter highly modified group and the more conventional lernaeopodids results in formation of two distinctly separate groups of species which appear to have arisen from ancestral stocks as parasites of marine teleosts, and may suggest two separate colonizations of the marine environment from fresh water with early marine bony fishes, the intermediate stages having been lost with their now-extinct hosts.

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## APPENDIX I. Haemolymph circulation in *S. californiensis*

Circulation of haemolymph in *Salmincola* is accomplished by the interaction of three interrelated pumping mechanisms, each of which may be observed in operation through the transparent cuticle of the live animal with a dissecting microscope. These are

(i) alternate contractions of the second maxillae

(ii) rhythmic lateral movements of the posterior mudgut

(iii) anti-peristalsis of the entire midgut.

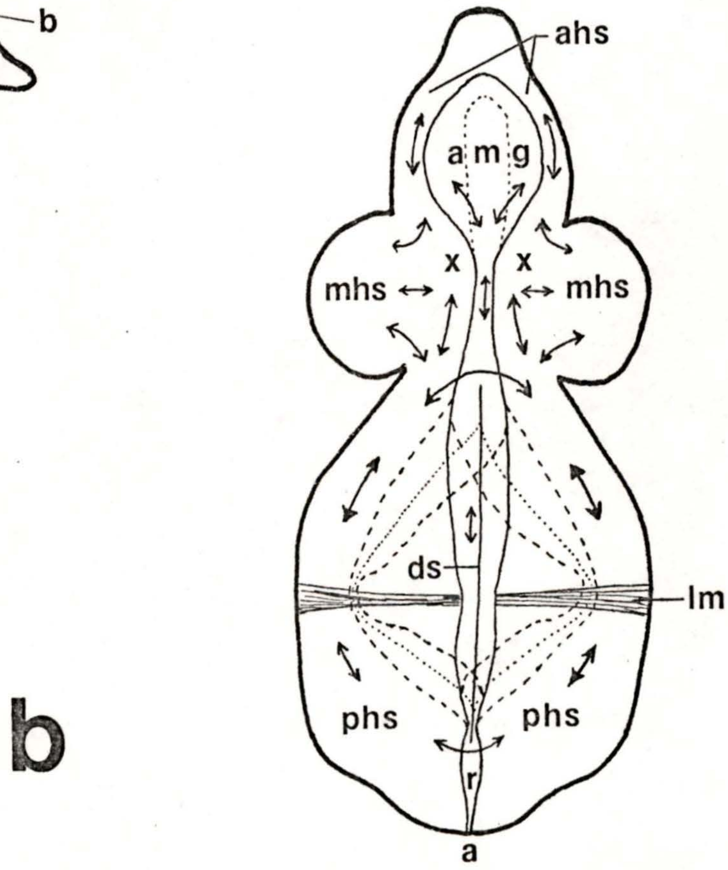
Each of these mechanisms will be described separately in turn, and then the functional interrelationships between them in providing complete circulation and mixing of the haemal fluid will be considered, with reference to Fig. 12. These mechanisms of haemolymph circulation have not been described previously in *Salmincola*.

(i) Alternate contractions of the second maxillae

The structure of the second maxilla in relation to the maxillary duct has been described in some detail earlier in this study. Also, brief mention was made of the haemal spaces within this appendage and their function in circulating haemolymph about the surfaces of the glandular epithelium surrounding the duct and the longitudinal muscle bands of the maxillae (Fig. 3). These haemal spaces may be regarded as tubular extensions of the cephalothoracic haemocoel, contractile along their long axis due to contraction of the longitudinal muscles. Contractions occur regularly, alternately and almost continuously in a healthy copepod, usually with a period of from 5 - 10 seconds. In addition to producing a back and forth movement of the fluid contained within the maxillary ducts from one maxilla to the other, via the bulla loop-duct as already described, these alternate contractions also produce

Fig. 12 Diagrammatic representation of the mechanisms and patterns of haemolymph circulation in *Salmincola californiensis*.

a - anus; ahs - anterior haemal space; amg - anterior midgut; b - bulla; ds - dorsal septum supporting posterior midgut; f - foregut; ge - glandular epithelium of maxillary duct; gt - glandular tissue dorsal to midgut between bases of second maxillae (function unknown); ld - loop duct of bulla; lm - lateral muscles of posterior midgut; lma - point of attachment of lateral muscles to midgut; md - second maxillary duct; mhs - maxillary haemal spaces; mt - mouth tube; phs - posterior haemal spaces; pmg - posterior midgut; r - rectum; va - valve at junction of foregut and posterior midgut; vs - ventral septum supporting posterior midgut; x - regions of mixing of haemal fluid from main body compartments. Arrows indicate directions of movement of haemal fluid due to actions of pumping mechanisms.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a counter-flow of haemal fluid back and forth within the haemal spaces of the maxillae. Each contraction causes a flushing of haemal fluid from the haemal spaces of the contracted maxilla into the cephalothoracic or "anterior trunk" haemocoel. At the same time extension of the opposite maxilla occurs due to expansion of its previously compressed haemal spaces by haemolymph expelled from the cephalothoracic haemocoel by displacement with fluid from the contracted maxilla. Hence a reciprocating pumping action of haemal fluid results, opposite in direction but coincident in time with the reciprocal movement of fluid in the maxillary ducts.

In addition, as noted previously, flow patterns of haemal fluid indicated by movement of small inclusions (haemocytes) suggest the presence of a system of valves at the base of each maxilla which direct at least a portion of the excurrent flow anteriorly toward the mouth tube and circumoesophagael ganglion, while incurrent flow appears to be drawn largely from more posterior regions of the anterior trunk haemocoel. This mechanism permits continuous circulation of both the maxillary duct "carrier" fluid and the haemal fluid surrounding these ducts within the second maxillae and also ensures continuous exchange of the latter with cephalothoracic haemal fluid by contractions of a single set of muscles, thereby providing an efficient means of transport to other parts of the body of materials absorbed from the host via the bulla and maxillary ducts.

(ii) Rhythmic lateral movements of the posterior midgut

The posterior midgut of Salmincola is that narrow tubular portion of the gut extending from the anterior end of the posterior

trunk haemocoel (the narrowing of the cephalothorax just posterior to the bases of the second maxillae) to the short rectum at the extreme posterior end of that cavity (Fig. 12). This portion of the midgut is supported and maintained in position within the maemocoel by dorsal and ventral membranes, which extend between the posterior midgut and the dorsal and ventral body walls throughout most of its length. The dorsal membrane is incomplete dorsal to the rectum, and both membranes are incomplete anterior to the aforementioned constriction of the cephalothorax immediately behind the bases of the second maxillae. These membranes thus form an incomplete dorsoventral partition separating the posterior trunk haemocoel into right and left halves throughout most of its length, the two chambers thus formed being in direct fluid communication only at their anterior and posterior ends.

The posterior midgut is also attached to the lateral body walls at a point slightly posterior to its midpoint by a pair of lateral muscle bands. These muscles normally contract alternately with a period of about 2 - 5 seconds in healthy copepods, giving rise to a vigorous (and easily visible) lateral back and forth movement or "swaying" of the posterior midgut and its supporting membranes (Fig. 12b). Consequently, a reciprocating two-way diaphragm pump is formed, each muscle contraction shifting the dorsoventral partition to right or left and causing the contained haemal fluid to flow alternately from one lateral chamber to the other via the anterior and posterior openings in the partition. A rapid and continuous circulation of haemal fluid about the reproductive organs and developing eggs contained within these chambers of the haemocoel is thus produced.

(iii) Anti-peristalsis of the entire midgut

Within the anterior cephalothoracic haemocoel (anterior to the bases of the second maxillae; Fig. 12) lies the anterior portion of the midgut, which is shorter than the posterior portion just described and also more nearly spherical than tubular in shape (when relaxed). The anterior segment is highly contractile, capable of expanding to almost fill the anterior haemocoel when relaxed or contracting to an almost tubular shape. Expansion and contraction occur in a regularly repeated sequence, with a period of about 3 - 5 seconds usually in healthy copepods, due to anti-peristalsis of the entire midgut. A wave of contraction starting at the posterior end moves anteriorly, forcing the fluid gut contents forward into the anterior portion of the midgut, which expands to become subspherical and occupy most of the volume of the anterior haemocoelomic cavity. Finally the anterior midgut contracts as the wave of contraction reaches it, forcing the contents back into the posterior midgut again, where the next wave of contraction is just beginning.

However, circulation of the fluid gut contents throughout the length of the midgut is only one consequence of anti-peristalsis, as this flow also provides the intermediate step in counter-circulation of haemolymph throughout the length of the trunk haemocoel. As the fluid within the gut is forced forward into the anterior portion of the midgut, an equal volume of haemal fluid is displaced from anterior to posterior trunk haemocoel by the expanding anterior midgut. When the anterior midgut contracts the reverse process occurs and haemolymph is forced forward again. The midgut and trunk haemocoel may thus be regarded as one closed fluid-filled vessel within another, such that

back for forth movement of fluid in the inner vessel by anti-peristaltic waves of contraction propogated along its muscular walls results in a similar but oppositely directed counter-flow of the fluid within the outer vessel.

Considered in combination, the anterior cephalothoracic haemocoel may be regarded as a mixing chamber for haemal fluid from the second maxillae (presumably carrying materials absorbed from the host) and haemal fluid from the posterior haemocoel (which bathes the reproductive tissues and developing eggs of the parasite). Some fluid leaving the maxillae is also passed anteriorly to irrigate the vital tissues in this region (e.g. the central nervous system). Haemolymph is pumped from the maxillae into the anterior haemocoel by alternate maxillary contractions and mixed with the remainder of the haemolymph by the counter-flow resulting from anti-peristalsis of the gut. The lateral movements of the posterior midgut and its supporting membranes form a further mechanism for circulating haemal fluid about the reproductive tissues within the posterior trunk haemocoel. The efficiency of these circulatory mechanisms in transporting materials from one part of the copepod to another, and particularly in distributing some materials absorbed via the bulla from the host can be considerable (e.g. $CN^-$ ion, $^{14}C$-amino acids and $^{14}C$-glucose in this study). It seems likely that the efficiency of this three-part circulatory system could be optimized by coordination of the timing of contractions of the various pumping mechanisms. Further studies into the precise timing of contractions and the interrelationships between these mechanisms are presently underway (Kabata; personal communication).

## APPENDIX II: Composition of U-$^{14}$C-amino acid mixture

The following table shows the approximate composition of the stock U-$^{14}$C-amino acid mixture and the final saline test solution, as taken from the ICN radiochemical analysis data sheet supplied with the stock solution.

| Amino acid | Mol.wt. | Stock solution | | Saline test solution | |
|---|---|---|---|---|---|
| | | % comp. (approx.) | sp.activity (approx.) | Concentration (approx.) | Activity (approx.) |
| Glycine | 75 | 4.0 | 74 mCi/mM | 2.67uM | 0.20 mCi/1 |
| L-alanine | 90 | 7.0 | 120 | 3.89 | 0.47 |
| L-Serine | 105 | 6.0 | 125 | 2.85 | 0.36 |
| L-Threonine | 119 | 3.5 | 165 | 1.47 | 0.24 |
| L-Proline | 115 | 2.3 | 185 | 1.00 | 0.18 |
| L-Valine | 117 | 14.5 | 155 | 6.20 | 0.96 |
| L-Leucine | 131 | 11.0 | 240 | 4.20 | 1.01 |
| L-Isoleucine | 131 | 8.5 | 230 | 3.24 | 0.75 |
| L-Phenylalanine | 165 | 8.5 | 340 | 2.58 | 0.88 |
| L-Tryosine | 181 | 9.0 | 345 | 2.49 | 0.86 |
| L-Aspartic acid | 133 | 4.5 | 160 | 1.69 | 0.27 |
| L-Glutamic acid | 147 | 4.5 | 210 | 1.53 | 0.32 |
| L-Lysine | 146 | 4.7 | 225 | 1.61 | 0.36 |
| L-Arginine | 174 | 9.0 | 260 | 2.59 | 0.67 |
| L-Histidine | 155 | 0.5 | 240 | 0.16 | 0.39 |

# APPENDIX III: Statistical analysis of $^{14}C$ uptake data

## A $^{14}C$-amino acids

The raw data ($^{cpm}/mg.$) may be summarized as follows:

| Group (n=20) | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Exposure time (hr) | 0 | 3 | 6 | 9 | 12 |
| Mean cmp/mg. ($\overline{Y}$) | 9.56 | 190.66 | 2630.42 | 2315.40 | 3033.50 |
| Variance ($s_y^2$) | $1.247 \times 10^2$ | $1.442 \times 10^4$ | $2.107 \times 10^6$ | $1.764 \times 10^6$ | $4.697 \times 10^6$ |
| St. dev. ($s_y$) | 11.17 | 120.10 | 1451.51 | 1328.02 | 2167.32 |

The variances as shown above are clearly dissimilar (heterogenous) and hence further analysis of variance cannot be performed on the raw data. The necessary condition of homogeneity of variances was met by logarithmic transformation of the raw data, and subsequent analysis of variance and comparison of group means were performed on the log-transformed data. Means were calculated using the logarithmic values and then back-transformed, as were 95% confidence limits to the mean in lieu of standard error.

| Group (n=20) | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Exposure time (hr) | 0 | 3 | 6 | 9 | 12 |
| Mean log cmp/mg. ($\overline{\log Y}$) | 0.707 | 2.183 | 3.331 | 3.233 | 3.275 |
| Variance ($s^2_{\log Y}$) | 0.251 | 0.097 | 0.096 | 0.175 | 0.260 |
| St. dev. ($s_{\log}$) | 0.501 | 0.312 | 0.310 | 0.418 | 0.510 |
| Mean cpm/mg. ($\overline{\text{antilog log y}}$) | 5.09 | 152.48 | 2140.87 | 1708.29 | 1882.80 |
| 95% confidence limits to mean | $L_1$=2.97 | 108.89 | 1534.32 | 1088.46 | 1087.22 |
| | $L_2$=8.73 | 213.31 | 2992.84 | 2681.09 | 3263.51 |

$$F = {}^{S^2 max}/_{S^2 min} = 2.708 < F_{max\ 0.05(5.20)} = 3.54 \text{ (n.s.)}$$

i.e. F not significant (P>0.05); variances are homogenous.

APPENDIX III cont'd

Analysis of variance:

$MS_1$ (among gruops) = 25.673 $MS_2$ (within groups) = 0.176

$F_s = {}^{MS_1}/MS_2 = 145.773$ *** $> F_{0.001(4.95)} = 5.05$

i.e. exposure via the bulla to the $^{14}C$-amino acid mixture in saline solution resulted in a highly significant (P<0.001) increase in $^{14}C$ content of S. californiensis body fluids and tissues over control values.

Subsequent a posteriori multiple comparisons among group means by the Student-Newman-Keuls LSR procedure (Sokal and Rohlf, 1969) indicated the following relationship between group means:

($\overline{Y}'$ = mean log $^{cpm}/mg.$; ** denotes statistical significance of the P<0.01 level):

$\overline{Y}'_{0\ hr.}$ ** < $\overline{Y}'_{3\ hr.}$ ** < $\overline{Y}'_{6\ hr.}$ ** = $\overline{Y}'_{9\ hr.}$ = $\overline{Y}'_{12\ hr.}$

B $^{14}C$-glucose

The raw data ($^{cpm}/mg.$) may be summarized as follows:

| Group (n=20) | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Exposure time (hr.) | 0 | 4 | 8 | 12 |
| Mean cmp/mg. ($\overline{Y}$) | 8.68 | 4591.82 | 4860.64 | 2548.62 |
| Variance ($S^2_y$) | 1.752x10 | $4.739 \times 10^6$ | $1.463 \times 10^7$ | $2.190 \times 10^7$ |
| St. dev. ($S_y$) | 4.19 | 2176.88 | 3824.37 | 1479.81 |

The variances are again clearly heterogenous, as with the $^{14}C$-amino acid data, and logarithmic transformation of the raw data is required to produce homogeneity of variances before further parametric statistical analysis can proceed. As previously, means and their 95% confidence limits were calculated from the log-transformed data, then back transformed.

| Group (n=20) | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Exposure time (hr.) | 0 | 4 | 8 | 12 |
| Mean log cpm/mg. ($\overline{\log Y}$) | 0.828 | 3.565 | 3.452 | 3.298 |
| Variance ($S^2_{\log Y}$) | 0.154 | 0.130 | 0.292 | 0.131 |
| St. dev. ($S_{\log Y}$) | 0.392 | 0.360 | 0.540 | 0.362 |

APPENDIX III cont'd

$F = {}^{S^2_{max}}/S^2_{min} = 2.25 < F_{max\ 0.05(4,20)} = 3.29$ (n.s.)

i.e. F not significant (P>0.05); variances are homogenous

Analysis of variance

$MS_1$ (among groups) = 34.291 $MS_2$ (within groups) = 0.177

$F = {}^{MS_1}/MS_2 = 194.065^{***} > F_{0.001(3,76)} = 6.010$

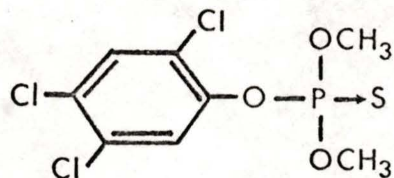
i.e. exposure via the bulla to o.1mM $^{14}C$-d-glucose in saline solution resulted in a highly significant (P<0.001) increase in $^{14}C$ content of S. californiensis body fluids and tissues over control values.

Subsequent a posteriori multiple comparisons among group means by the Student-Newman-Keuls LSR procedure (Sokal and Rohlf, 1969) indicated the following relationship between group means ($\bar{Y}'$ = mean log ${}^{cpm}/mg.$; ** denotes statistical significance at the P < 0.01 level):

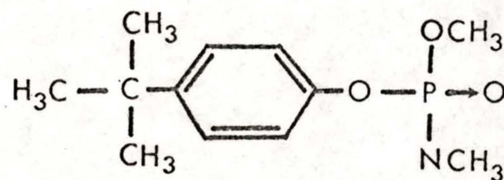
$$\bar{Y}'_{0\ hr.}\ {}^{**} < \bar{Y}'_{4\ hr.}\ {}^{**} = \bar{Y}'_{8\ hr.} = \bar{Y}'_{12\ hr.}$$

APPENDIX IV. Chemical structures of some compounds referred to in the text

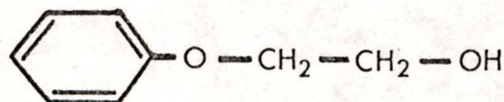
A. Potential chemotherapeutic compounds




Ronnel; 0,0-Dimethyl 0,-2,4,5-Trichlorophenyl phosphorothioate (Mol. Wt. 321) (Spencer, 1968).

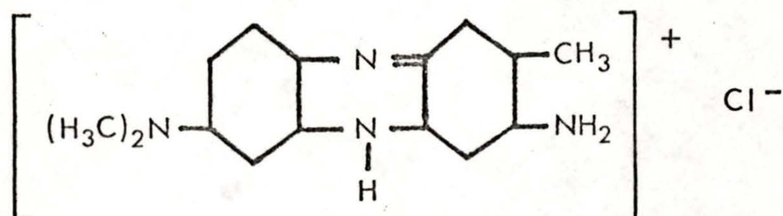



Ruelene; 4-tert-butyl-2-chlorophenyl methyl methylphosphoramidate (Mol. Wt. 292) (Spencer, 1968).

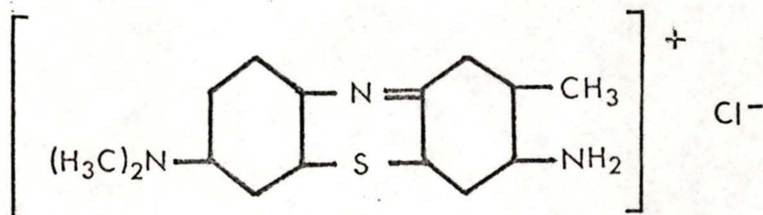



Phenoxethol; =2-phenoxy ethanol (Mol. Wt. 138) (Stecher, 1968).

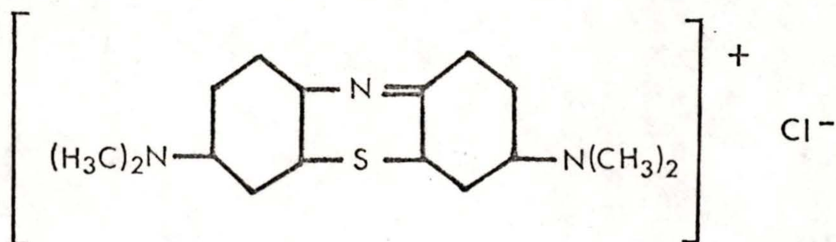
B. Vital stains




Neutral Red (Mol. Wt. 289) (Gurr, 1965).



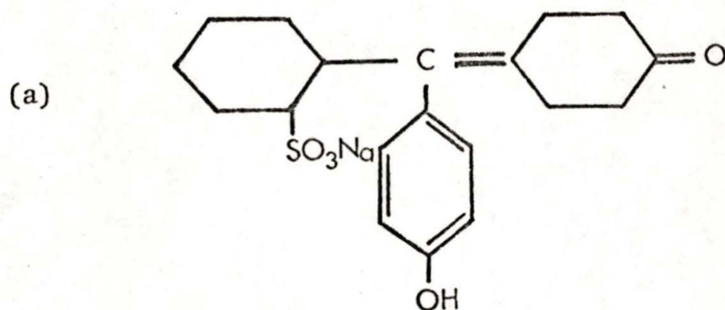

Toluidine Blue (Mol. Wt. 306) (Gurr, 1965).




Methylene Blue (Mol. Wt. 374) (Gurr, 1965).

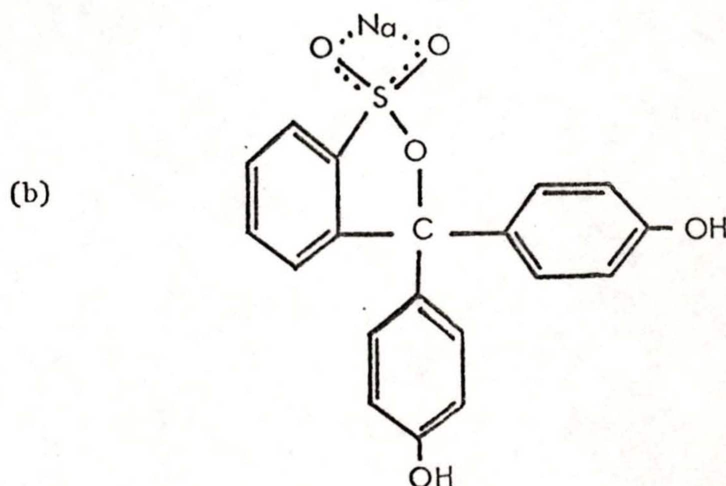
Vital stains (cont'd)

(a)

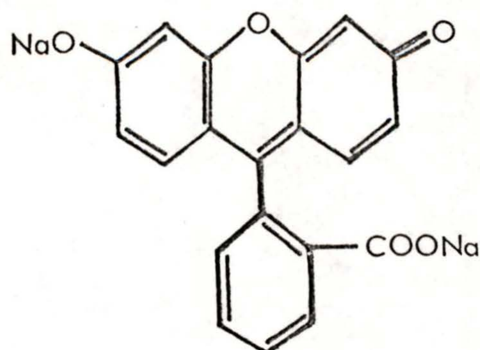



OR

(b)




Phenol Red (Mol. Wt. 376); (a) from Gurr (1965), (b) from Stecher (1968).




Uranin; fluorescein disodium salt, water soluble (Mol. Wt. 376) (Gurr, 1965).

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Kabata, Z. and B. Cousens. 1977. Observations on host-parasite relationships between sockeye salmon, Oncorhynchus nerka, and Salmincola californiensis (Copepoda: Lernaeopodidae). J. Fish. Res. Bd. Canada 34: 191-202.

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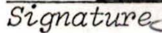
TITLE OF THESIS:

Structure and function of the bulla, second maxillae and maxillary ducts of Salmincola californiensis (Dana, 1852) (Copepoda: Lernaeopodidae).

AUTHOR:


*Signature*

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24 / Aug. / 1977.
*Date*