Morphological And Cytochemical Studies On The Skin Of Rainbow Trout, Salmo Gairdneri, Richardson

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To my husband Zé, who so beautifully held hearth and home together during my absences and to my children Ana and Zé

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ABSTRACT

A series of studies concerning the structure and ultrastructure of the skin of rainbow trout was carried out, especially associated with growth and sexual maturation. An increase in the thickness of the dermis and epidermis and a decrease in the number of mucous cells were noted in the males, together with an infiltration of mononucleated cells in both sexes during sexual maturation. The structure of the gills and thymus was also examined.

In the ultrastructural studies special attention was paid to lymphocyte-like and macrophage-like cells, which were interpreted as infiltrating elements. Presumed macrophages, with or without melanin granules, were seen crossing the basement membrane, which reinforced the idea of a dermal-epidermal traffic.

Phosphotungstic-acid staining was applied to ultrathin sections of the epidermis to evaluate the distribution of complex carbohydrates. Among other localizations, positive staining was observed in mucous cells and in the membrane of large vesicular bodies in macrophages and filament-containing cells. The possibility of such structures being lysosomes is discussed.

Immunoglobulin-containing cells were located in the skin through immuno-cytochemical methods, PAP and immunogold labelling, in semithin and ultrathin sections. Rabbit serum anti-trout Ig was used and the details of its production are given.

In the epidermis, lymphocyte-like cells showed a degree of intracytoplasmic labelling, but this was not sufficient to draw conclusions as to whether these cells were antibody-producing or not. Some mucous cells also stained positively.

To evaluate how bath vaccination influenced the production of antibody in the skin, a group of juvenile fish were vaccinated anti-Vibrio anguillarum. Immuno-cytochemical studies were undertaken, using an immuno-sandwich technique, to locate specific antibody anti-Vibrio anguillarum.

Finally, a discussion is presented of the involvement of the skin in specific immune reactions. It seems to be a tissue fully equiped for antigen-trapping, where mucous cells could be responsible for the transport, or even the synthesis, of antibody into the skin surface, to be incorporated into the mucus.

The lack of protection shown by the skin of sexually mature males of certain salmonid species is considered and thought possibly due to the decrease in mucus production under hormonal influence and thereby a decrease in available immuno-globulins at the body surface.

INTRODUCTION

The problem of controlling diseases of fish is certainly as old as the first attempts at creating modern aquaculture systems. The experience of intensive breeding of other animal species has provided useful parallels, demonstrating that prophylaxis is the best way to maintain a healthy stock. Attempts at immunizing fish have become a prolific field of research. Vaccination attempts were undertaken as long ago as the beginning of the century, as is cited by Ridgway, Hodgins and Klontz (1966) in their review work on the immune response in teleosts. These first experiments used bacteria in order to test the production of agglutinins in some species of teleost fish. In other reviews such as those of Finn (1970), Snieszko (1970) and Corbel (1975) it is noticeable that there is a much greater interest in fish immunology, not just intending to improve protection against diseases, but also in order to better understand the phylogeny of the immune response.

Recent publications, including two books that collected the work presented in recent conferences on fish immunology (Van Muiswinkel and Cooper, 1982 and Manning and Tatner, 1985), have shown that most of the research work on fish immunization involves bacteria such as Vibrio anguillarum and Aeromonas salmonicida, and the species most widely used in these studies are the salmonids, especially rainbow trout (Salmo gairdneri Richardson).

In spite of the large amount of knowledge collected on this subject, there is a number of questions which remain unanswered or quite controversial. In fact, most attention has been directed towards the humoral immune response of the fish to vaccination. Their degree of protection has been principally measured by mortality rates upon challenge, or by levels of serum agglutinins of immunized animals. However, in numerous circumstances there was poor correlation between the degree of protection and the agglutinin levels measured.

In 1942, Duff, in the first successful oral immunization of rainbow trout against Bacterium salmonicida, found very low antibody levels in the serum and in some individuals, no agglutinins were detected at all. However, this seems to be common in orally immunized fish (Fletcher and White, 1973; Gunnels, 1976). In 1977, Croy and Amend compared two procedures, hyperosmotic infiltration and intraperitoneal injection, to vaccinate sockeye salmon (Oncorhynchus nerka) against vibriosis, and obtained excellent protection with hyperosmotic infiltration, although the antibody titres never exceeded 1 : 8. Gould, O'Leary, Garrison, Rohovec and Fryer (1978), in their studies on spray vaccination, a novel vaccine delivery system, also found very low serum antibody titres against Vibrio anguillarum although the fish were protected. Smith, McCarthy and Paterson (1979) showed that brown trout (Salmo trutta) orally and parenterally vaccinated against Aeromonas salmonicida did not have levels of circulating antibodies significantly different from those of control fish. Palmer and Smith (1979) also vaccinated Atlantic salmon against Aeromonas salmonicida by hyperosmotic infiltration and intraperitoneal injection and concluded that low serum agglutinin titres did not necessarily mean lack of protection against challenge. Similar statements were made by other authors working on vibriosis vaccination (Baudin-Laurencin and Tangtrongpirus, 1979; Rosenkvist - Jensen, 1982).

The opposite situation was found by investigators such as Klontz (1969, cited by Snieszko, 1970) who used different methods to immunize brook trout (Salvelinus alpinus). The fish that had been parenterally immunized with heat-killed Aeromonas salmonicida had the highest degree of precipitating antibody, but none of the trout were protected against furunculosis. Michel (1979) also immunized rainbow trout against furunculosis and the use of intraperitoneal vaccination resulted in an elevation in circulating antibodies, but no protection against challenge was observed. Therefore, it seems reasonable to assume that serum agglutinin titres are not necessarily a reliable index of immunity.

The present knowledge on how the cell-mediated immune mechanisms are involved in fish vaccination is very limited, but receiving some attention. Smith et al. (1979), in their previously referred to work on brown trout vaccination against Aeromonas salmonicida studied the cellular immune response using a modified leucocyte migration inhibition test. They found a close association between disease resistance and cell-mediated immunity.

Anderson, Roberson and Dixon (1979) used methods to detect the levels of splenic antibody-producing cells in order to evaluate the response of rainbow trout to immunization with antigens extracted from Yersinia ruckeri and Aeromonas salmonicida. The exposure was performed by three methods: intraperitoneal injection, immersion in the antigen preparation or addition of such a preparation to the holding water. They found the highest number of antibody producing cells in the spleen of fish treated by the third method, but they stated that they ignored whether the immunization method had any influence on antigen uptake.

Gosting, Mirando and Gould (1981) used an immunocytoadherence assay to monitor the response of antigen-binding cells in the peripheral blood of sockeye salmon after immunization with Vibrio anguillarum bacterin. They found an elevated response in less than one day, both in intraperitoneally injected fish and in fish that had been immersed in the bacterin. The cellular immune response of rainbow trout vaccinated by immersion and injection with Vibrio anguillarum was also tested by Sakai, Aoki, Kitao, Rohovec and Fryer (1984). Plaque-forming cells were rarely detected by these authors in the anterior kidney and spleen of trout immunized by immersion, but reached significant levels in the organs of the parenterally immunized fish. Rosette -forming cells, however, increased rapidly in the anterior kidney and spleen of the trout vaccinated both by immersion and intraperitoneally. These authors also noticed that the serum antibody titre of the fish vaccinated by immersion was as low as in the non-vaccinated fish.Further investigations into cell-mediated immunity would appear to be more necessary,

particularly concerning the response to various vaccination procedures.

Another controversial aspect of fish vaccination concerns the entry of the antigens during hyperosmotic infiltration, direct immersion or spray vaccination. These methods seem to be the only economical and effective ways to deliver a vaccine to a large number of fish. Although some methods exist to determine how much antigen actually penetrates the fish during direct immersion (Tatner and Horne, 1984), the mechanism of antigen uptake has not yet been determined with certainty. The first experiments on hyperosmotic infiltration in rainbow trout by Amend and Fender (1976) showed that bovine serum albumin (BSA) was absorbed into the blood stream. They suggested that the lateral line was the primary route of BSA entry into the fish. In a paper published in 1978, still concerning hyperosmotic infiltration, Fender and Amend excluded the gills as a major entrance area for BSA, because an increase in the functional area of the gills did not correspond to a higher uptake of antigen. By contrast, a reduction was noticed upon stimulation with epinephrine. On the other hand Bowers and Alexander (1981) and Alexander, Bowers, Ingram and Shamshoom (1982), who undertook a similar series of in vivo and in vitro experiments, concluded that the portal of entry of viable bacteria into trout during hyperosmotic infiltration was the gills.

Tatner and Horne (1983) in their studies on the factors influencing the uptake of Vibrio anguillarum vaccine in direct immersion experiments with rainbow trout, reported that the site of entry of the vaccine appeared to be concentrated in the head region. They also commented that the differences between the results reported by previous authors could be due to the nature of the antigens used, as the BSA was a soluble protein and the bacteria particulate.

The fate of V. anguillarum bacterin delivered by intraperitoneal injection, immersion and by oral route was studied in rainbow trout by Nelson,Rohovec and Fryer (1985), who concluded that with the first two immunization methods the

bacterin entered the circulation system. Based on such observations these authors suggested that the immersion bacterins did penetrate the epithelial integument, although they were unable to confirm such a hypothesis.

Not a great deal of attention has been paid to the skin by researchers involved in vaccination studies. However, the presence of antibodies in the skin mucus has already been shown for a variety of species. An early observation on the presence of antibodies in fish mucus was made by Hildemann (1962), whose observations on the relationship of the Amazonian Symphysodon discus parents and newly hatched fry, led him to suggest that the young fish would normally obtain antibodies against pathogens from the parental skin mucus, which would be functionally equivalent to the mammalian colostrum. In 1961, O'Rourke had already showed by immunodiffusion technique, that some of the serum protein antigens in fishes were also found in the mucus. Following the parenteral immunization of plaice (Pleuronectes platessa) with several antigens, Fletcher and Grant (1969) showed the presence of antibodies in the surface mucus. They also advanced the idea of the involvement of a specific secretory system.

Di Conza (1970) studied the serum and mucus of the catfish Tachysurus australis and found that all body mucus samples contained potent haemagglutinins, but they seemed to belong to a different molecular species from the natural serum haemagglutinins. He later obtained further support for this view following immunization of catfish by intraperitoneal and intramuscular injections of BSA, by failing to detect specific antibody activity in the mucus of immunized fish (Di Conza and Halliday, 1971). These results suggested that the immunoglobulins found in the mucus would be locally synthetized, rather than be derived from the blood.

Bradshaw, Richard and Sigel (1971) also detected haemagglutinating activity in the normal mucus of the gar (Lepisosteus platyrhincus), and this activity was enhanced by immunization with sheep red blood cells. Nevertheless, they failed to

detect agglutinins for Salmonella typhosa H antigen in the mucus of fish which had been intramuscularly immunized. These results led them to suggest that the agglutinating activity in the mucus was different from that in the serum.

Harrel, Etlinger and Hodgins (1976) demonstrated the presence of anti-Vibrio anguillarum agglutinins in the body mucus of intraperitoneally immunized rainbow trout. Such antibodies were indistinguishable from serum immunoglobulins by immunodiffusion and immunoelectrophoresis. These results corroborate the findings of Fletcher and White (1973), who also detected antibodies in the mucus of plaice parenterally immunized with the same Vibrio anguillarum antigens. Similar findings were reported by Ourth (1980), who found agglutinating antibodies against Salmonella paratyphi in the mucus of channel catfish (Ictalurus punctatus), intraperitoneally injected with a suspension of killed bacteria .

Sheep red blood cells were also used as antigens in the experiments of St. Louis-Cormier, Osterland and Anderson (1984). The cells were injected into the peritoneal cavity of rainbow trout and, subsequently, agglutinating antibodies were detected both in the mucus and serum of the sensitized fish.

In all the experiments mentioned so far, the antigenic stimulation was systemic. However, the concept of antibody production induced by antigenic stimulation of mucosal surfaces has long been known for mammals, resulting in local antibody production (Bourne, 1976). The skin of fish is in fact a mucosal tissue, with a stratified epithelium, where a variable number of unicellular mucous glands are interspersed. It seems reasonable to expect that, at least in some aspects, the fish skin will show similarities with the mammalian mucosal surfaces.

The studies of Lobb and Clem (1981 a , b, and c) on the immunoglobulins (Igs) of serum, mucus and bile of the fish Archosargus probatocephalus, showed that the Igs found in the cutaneous mucus and bile were not due to transudation or active

transport from the serum. Furthermore, they identified an approximately 95,000 dalton protein, linked to low molecular weight immunoglobulins of the skin mucus They thought that such a protein might be the teleost equivalent of the mammalian secretory component (Lobb and Clem, 1981 b).

Such secretory component, in mammals, is a protein synthesized by epithelial cells and present on the surface of the cell in which it was produced. The mammalian dimeric IgA binds strongly to the secretory component and the complex is actively endocytosed, transported across the cytoplasm and secreted into the external body fluids (Roitt, 1980).

There do not appear to be any reports on antibody identification in the surface mucus of fish immunized by bath immersion, spray vaccination or hyperosmotic infiltration. Nevertheless, the question of a skin trapping system for antigens seems to be clearly possible following the studies of Smith (1982). The uptake of particulate and nonparticulate antigens was studied by this author, who immunized rainbow trout by hyperosmotic infiltration or bath methods, employing either a range of radio labelled proteins, or latex particles sensitized with radiolabelled proteins. He concluded that the uptake was greatly increased when the antigen was in particulate form, suggesting the existence of mechanisms of active trapping.

A recent study on antigen uptake mechanisms in rainbow trout following spray vaccination was undertaken by Hockney (1985). His results were not very conclusive, although he located antigen within epithelial cells six hours after the spray exposure.

In mammalian skin, the Langerhans cells present in the epidermis are antigen-trapping cells (Shelley and Juhlin, 1976), capable of substituting macrophages in antigen presentation (Braathen and Thorsby, 1980). In fish skin, a reference was made by Mittal, Whitear and Agarwal (1980) to the presence of

intracellular granules in epidermal cells of Monopterus cuchia, which resembled the typical granules of the mammalian Langerhans cells.

In spite of the scanty references in the literature to the presence of Langerhans cells in the fish epidermis, a large body of information can be collected referring to the presence of other immune-related cells, such as lymphocytes and, more rarely, macrophages. The presence of lymphocytes in the epidermis of non-salmonid teleosts has been shown by light microscopy (e.g. Percy, 1970; Mittal and Munshi, 1971, 1974; Bullock and Roberts, 1974; Logan and Odense, 1974; Mittal and Banerjee, 1974, 1975, 1976; Banerjee and Mittal, 1975; Mittal, Agarwal and Banerjee, 1976; Zaccone, 1979). Electron microscopical studies also demonstrated the presence of cells with the morphological characteristics of lymphocytes in the epidermis of several species of teleosts (e.g. Brown and Wellings, 1970, Leonard and Summers, 1976; Phromsuthirak, 1977; Mittal and Whitear, 1979; Mittal et al. 1980; Ferri, 1983a).

In salmonids, the lymphocytes were described by some authors as round cells with small, darkly staining nuclei (Roberts, Shearer, Elson and Munro, 1970; Pickering and Macey, 1977). Pickering and Richards (1980) described lymphocyte--like cells in the epidermis of the brown trout. Such findings were confirmed in studies on the ultrastructure of the skin of rainbow trout carried out by Peleteiro and Richards (1985). The presence of macrophages in the epidermis was also noted by these authors, although only a superficial reference was made to such cells as attention was mainly directed in that work towards the presence of lymphocytes.

References to macrophages or histiocytes in the skin of teleosts are quite rare. Percy (1970) described macrophages as amoeboid non-granular cells in the epidermis of the gold fish. Brown and Wellings (1970) also reported the presence of histiocytes in the epidermis of Hippoglossoides elassodon, a pleuronectid fish.

Roberts, Young and Milne (1971) gave a detailed description in the skin of plaice

of macrophages, actively migrating out to the periphery of the epidermis, excreting melanin into the mucus. Bullock and Roberts (1974), in their work on the dermatology of marine teleosts, also referred to macrophages in the epidermis. Acid phosphatase-positive epidermal macrophages were described by Phromsuthirak (1977) in his electron microscopy studies on the skin of stickleback (Gasterosteus aculeatus).

Further investigations on the role of the immune related cells in the skin of teleosts were carried out in the present studies.

Sexually mature fish are a particularly good source of material for structural observations on the skin. It is well established that sexually maturing salmonids undergo extensive changes in the skin (Smirnov, 1959; Pickering, 1974, 1977; Wilkins and Jancsar, 1979). Furthermore, Pickering and Richards (1980) described increased number of lymphocyte-like cells in the epidermis of spawning fish.

Immunoglobulin-containing cells have been identified in the epidermis of rainbow trout by immunofluorescence (St. Louis-Cormier et al. 1984) and by immunoperoxidase methods (Peleteiro and Richards, 1985). In both cases, only light microscopical methods were used, and cell structure details of the positive cells were not visible, particulary using the immunofluorescence method.

More observations on the presence of immunoglobulins in the epidermis are therefore needed, in normal, fully developed fish, as well as in immunized specimens and the question of how direct immersion vaccination techniques affect the structure of the skin still requires answering.

CHAPTER 1

LIGHT MICROSCOPY OBSERVATIONS OF THE SKIN OF RAINBOW TROUT

As stated in the general introduction, the observation of the skin of salmonids has been the object of study of several authors. However, the large number of different species within this group makes such observations sometimes controversial, with differing results dependant on the species examined.

This chapter represents the results of histological examinations of the modifications taking place in the skin and especially the epidermis, during normal growth and development of rainbow trout. The differing structure shown by this tissue indicates its enormous capacity of adaptation to environmental conditions and also to internal determinants of the animal itself, such as sexual development.

1.1 - MATERIALS AND METHODS

Adults - Twenty adult fish, 7 males and 13 females, were used in the present study. They were obtained from the following sites:

1) POSTO AQUICOLA DE MANTEIGAS (Serra da Estrela, Portugal)

Two males (3 + and 5+) and four females (two 3+ and two 5+), weighing between 500 and 900 grammes, maintained as part of a broodstock for restocking purposes, were kept in fresh water, with an annual temperature range of 5 to 11°C. The site is government owned and is located on the eastern slope of the highest Portuguese mountain, at approximately 1000 m altitude. The animals were kept in concrete tanks, and fed on a commercial pelleted diet.

Both the males and females were obtained during the sexual maturation period of this species, which occurs in Portugal between late December and mid January. The females were at the "ripe" stage, with the abdominal cavity filled with fully

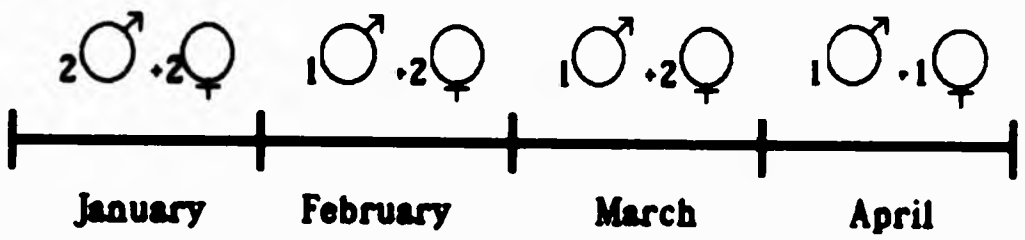
developed eggs. The males had the abdominal cavity full of milt, which was running easily at the slightest pressure.

All animals were in good general health, no lesions having been observed at necropsy. The skin also appeared grossly normal, with the usual pigmentation distribution characteristic of this species. The males had a protruding lower jaw, an indication of their advanced sexual development.

2) TRUTURÃO - PRIVATE TROUT FARM IN POMBAL, WEST CENTRAL PORTUGAL

Five males and seven females were obtained from this private trout farm, that uses fresh-water from an underground river, whose source is located 400 metres from the farm. The water temperature ranges from 12 to 18°C throughout the year.

Both males and females were aged between 3+ and 4+, weighing between 400 and 750 grammes. They had been used for breeding the year before. The sampling process occurred during the first four months of 1984, distributed as follows:



In February 1985, another male (4+) and a female (4+) were sampled. At the farm, the fish were kept in concrete tanks and fed on a commercial pelleted diet from SAPROPOR (Portugal).

All animals sampled during the spawning season, or up to two months after (February, March), had their abdominal cavity full of sexual products, eggs or milt, as none of them had been used for reproduction in 1984 or 1985. The farmers were selling their broodstock for human consumption, as they had changed their policy from producing their own eggs to buying in eyed ova.

The group of fish sampled in February and March were used in immunization experiments (see chapter 4) and were brought to the Veterinary School in Lisbon (Escola Superior de Medicina Veterinária), where they were kept in a recirculating water system in plastic tanks (figure 1) for a period varying from two to six weeks. The water in the system was maintained at a relatively constant temperature of around 11°C. During this period they were fed on the same diet as at the farm.

The state of health of these adult rainbow trout was considered good, although some animals, especially the males, showed signs of prior skin injury, with healed lesions showing loss of pigment and scar formation. One of the females brought to the Veterinary School died with severe Saprolegnia lesions (figure 2). Such lesions were left to progress, no treatment having been carried out.

Juveniles - All fish were obtained from the second sampling site, at TRUTURÃO. Eighteen juvenile rainbow trout, weighing between 70 and 200 grammes were sampled. As they were also used in a vaccination experiment (see chapter 6) they were brought into the Veterinary School in Lisbon, where they were maintained for a period varying between 3 days to seven weeks. The facilities were the same as those used for the adults, a recirculating water system, using plastic tanks. They were fed on the same diet as the farmed fish. Two of them developed fungal lesions while in the Veterinary School, which were treated with malachite green. Apart from these cases, all fish were in good condition, and none of them displayed signs of sexual maturity at necropsy.

Fry - 4-week and 8-week old rainbow trout fry were also obtained from TRUTURÃO. They were used for histological studies of the skin, as a comparison of the tissue's evolution through the growing process.

Sampling methods used for all fish:

To facilitate sampling, especially for further electron microscopical

examination, the fish were anaesthetised in tricaine methane sulphonate (MS 222, Sandoz) at a concentration of 100 ppm.

Samples of skin were usually obtained from four areas (dorsal, ventral, caudal and head region) for the adults and lateral and head region for the juveniles (figure 3). Other organs were also occasionally sampled, such as gills, liver, spleen, ovary, testis and thymus. Samples were immediately fixed in 10% phosphate buffered formalin and after 24 hours fixation they were then embedded in paraffin wax and 5 µm sections obtained using a Leitz rotatory microtome.

The sections were stained with haematoxylin and eosin (H & E), periodic acid--Schiff (P.A.S.) and toluidine blue (see appendix for details on the methods).

1.2 - OBSERVATIONS

A. GENERAL STRUCTURE OF THE SKIN

The organization of the skin of rainbow trout, although undergoing some changes throughout the growing process, showed the same basic pattern in all age groups. It is distinguishable from mammalian and avian skin mainly by the presence of mucous cells in the epidermis and the large number of melanin--containing cells in the superficial dermis, just beneath the basement membrane. The skin was obviously divisible into dermis and epidermis, both layers varying in thickness, according to the age and to the sexual development of the fish. Connection with the muscle or periosteal tissue was made through the hypodermis of loose connective tissue.

In the very young fry the dermis was restricted to a continuous sheet of melanin-containing cells, with an obvious basement membrane separating it from the epidermis (figure 4). The latter consisted of five to eight strata of epidermal cells, depending on the area observed. The presence of mucous cells was conspicuous in the median and upper layers, although they seemed smaller than the same cells in the older animals. No particular organization was noticed amongst the epidermal cells. They assumed the same size and shape along the total thickness of the tissue. The muscle myomeres were visible just beneath the thin dermis. No scales could be seen at this stage of development.

The skin of very young juveniles ($\pm$ 30 weeks) showed marked differences such as a wider dermis with small scales within scale beds (figure 5), except in the head skin, where they do not form. Bundles of collagen fibres, with interspersed fibroblasts, could be seen parallel to the surface of the skin. The width of the epidermis did not seem to increase significantly, although the mucous cells appeared to be slightly larger. A second layer of melanin-containing cells could be

seen in the hypodermis.

In the juvenile fish, with a total length varying between 15 to 20 centimetres, the structure of the skin was basically the same as in the adults (figure 6). In the dermis, two different areas were regularly identified: an upper layer of loosely arranged tissue, with many pigment cells, and a deeper one, with thick collagen bundles. The first layer, usually referred to as stratum spongiosum, showed a continuous sheet of melanin-containing cells, especially in the dorsal or head skin. In some other areas the density of these pigmented cells was lower, such as in sections of ventral skin (figures 7 and 12) , or in areas where inflammatory lesions were present (figure 16). The overlapping scales were another obvious feature of the stratum spongiosum, which projected occasionally into the epidermis and resembled pockets of dermal tissue. The underlying stratum compactum was formed by orthogonally arranged collagen fibres, with few interspersed cells between them. In the head skin, the stratum spongiosum was quite narrow, mainly represented by the pigmented cells, without scales, and an extremely thick stratum compactum occupying most of the total width of the dermis (figure 8).

The thickness of the epidermis varied greatly depending on the part of the body examined. The organization of the epidermal strata did not seem so evident in the juvenile as in the adults. The basal layer, immediately adjacent to the basement membrane, was formed by tall columnar cells. This layer was not so clearly visualised in some of the small juveniles. In the median layers, the epidermal cells were more irregularly arranged, assuming a rounded or polygonal shape. Their nuclei, moderately dense and with no obvious nucleolus, were usually rounded, except in the basal cells, where they accompanied the cell shape, showing an elongated appearance. As the epidermal cells approached the surface, they became flattened, with equally horizontal nuclei.

The mucous or goblet cells were easily identified, as outstanding large clear

cells, whose localization was restricted to the median and upper layers. Their nuclei were always pressed against the basal pole by the cell contents. The mucus staining properties were not always the same. In the haematoxylin-eosin stained sections the goblet cells were usually very clear and unstained (figure 9) though sometimes they stained more deeply (figure 10), eventually assuming a marked blue colour (figure 7). All the mucous cells stained magenta with the P.A.S. technique.

Occasionally, melanin granules could be seen in the epidermis, although it was difficult to see if they were inside or outside any of the cells (figure 6).

Whenever comparing this general skin structure between different specimens of adult fish, major changes could be noticed, especially in the mature males during the breeding season. A marked increase in the dermal and epidermal thickness usually occurred, together with a pronounced folding of the basement membrane. Such changes were particularly noticeable in the dorsal skin.

As a consequence, the dermis seemed to form deep papillae, penetrating the epidermis up to its surface (figure 11). The scales, due to the dermal development, looked as if deeply buried within the tissue. In some of the males, the frequency of mucous cells in the epidermis seemed markedly diminished (figure 11). However, other males showed increased numbers of such cells (figure 12). In the females, these modifications were not so pronounced, although a slight thickening of the epidermis seemed to occur during the breeding season (figure 9).

Infiltrating the epidermis, small cells without distinguishable cytoplasm, but very darkly staining nuclei, were frequently seen. Sometimes they appeared in large numbers, just above the basal cells and almost forming an individual cell layer (figures 8, 9 and 10). Such small cells were usually surrounded by a clear space. In areas where they appeared in groups, the spaces seemed to fuse, forming large clear areas, separating the epidermal cells (figures 12 and 13). They did not

seem to be restricted to any sexual or age group. Even in the epidermis of juvenile fish these cells were frequently identified (figure 10). In a few cases, small cells with darkly stained nuclei were also observed in large numbers in the dermis, just beneath the basement membrane. Occasionally, even this latter structure seemed to become infiltrated (figures 8 and 9).

Although not restricted to any age group, as mentioned above, these small cells were more frequent in the epidermis of adult fish, during their sexual maturation phase. During this same period, there appeared to be a higher degree of surface cell sloughing, particularly in the females. Sometimes, the upper epidermal cells appeared rounded and clear, without assuming the characteristic flattened profile (figure 14). On other occasions, groups of epidermal and goblet cells seemed to detach from the superficial layer.

In most skin sections, no intercellular spaces were noticed between epidermal or mucous cells. However, cases of spongiosis, identified through the presence of intercellular spaces, were observed, although infrequently (figure 15).

In one particular case of spongiosis of an adult female fish epidermis, the malpighian cells had undergone a most unusual change. Their cytoplasm was filled with large eosinophilic granules (figure 16). At high magnifications these granules were obvious individually around apparently normal nuclei (figure 17). The spongiosis was particularly severe and no mucous cells were identified in this affected epidermis. Small cells with dark nuclei could be seen interspersed between the modified epidermal cells. A major infiltration of mononucleated cells and neutrophils occurred in the corresponding dermis (figure 16).

The skin sections of the female which died with severe Saprolegnia lesions, showed complete destruction of the infected area's epidermis together with fungal hyphae (figure 18). A microbial infection was also present with many bacteria

visible in the remainder of the upper dermal layer. In some areas, the bacteria had penetrated into the muscle with signs of myositis, such as infiltration of inflammatory cells together with lesions of the muscle fibres (figure 19).

B. STRUCTURE OF THE GILL

Observation of the gills showed that in both the juveniles and the adults the general structure was the same, with the branchial arch covered by its stratified epithelium, very similar to the epidermis, endowed with numerous mucous cells (figure 20). Underneath the epithelium, the supporting connective tissue, with a rather loose structure, showed eosinophilic granular cells as well as mononucleated cells with characteristics similar to macrophages and lymphocytes. Fat cells were also seen. The epithelium of the branchial arch was frequently infiltrated by the same small cells with dark staining nuclei, already described for the epidermis (figures 20 and 21). This fact was observed in adults and juveniles, with marked individual differences. Occasionally, distinctive cellular aggregates could be seen assuming an arrangement similar to taste buds (figure 21). Projecting from the arch were the primary lamellae, also covered by a mucoid epithelium. The secondary lamellae showed up in sections as delicate folds, looking like thin branches of the primary lamellae. The epithelium of this latter structure showed frequent infiltrations throughout its length and at the base of the secondary lamellae by small cells with dark staining nuclei, as was described for the branchial arch and for the epidermis (figure 22). The infiltration was most marked at the tip of the primary lamellae (figure 23).

C. OTHER ORGANS

The microscopical observation of other organs showed no significant deviation from the normal structure.Attention was paid to the lymphoid organs, namely the

thymus, that showed a particular development in the young fry and in the juveniles. Histologically, this organ was formed in fry by a compact lymphoid tissue (figure 24), covered by a single layered epithelium with sparse mucous cells, that separated the organ from the gill cavity, and by a very thin layer of connective tissue, that isolated it from the underlying muscle. In between the thymocytes, a particular cell type could be seen , with large pale nucleus (figure 24). The cytoplasm of such cells was hardly visible because of the high density of thymocytes.

The thymus in the juveniles was much more developed (figure 25) showing a high concentration of thymic cells in the periphery of the organ, although no clear limits could be established from the central area, where cell density seemed lower. Small septa of connective tissue were regularly seen throughout the organ. The same cells with large clear nucleus already noticed in the thymus of fry, were regularly found with no particular distribution within the organ, but not associated with the connective tissue septa. The epithelium which separated the organ from the gill chamber, showed an increased number of mucous cells compared to the same tissue in the fry.

1.3 - DISCUSSION

The light microscopy observations on the skin of rainbow trout described in this thesis generally agree with other authors' descriptions on the skin of salmonids (Roberts et al. 1970; Pickering, 1974, 1977; Harris and Hunt, 1975a; Pickering and Macey, 1977; Pickering and Richards, 1980). They also agree and follow on from previous observations on the structure of the skin of mature rainbow trout made by the present author (Peleteiro, 1981; Peleteiro and Richards, 1985). In the present work these observations have been extended to fry and juvenile fish, in order to evaluate the development of the skin during the growing process. It was clear from the results presented here that no significant changes could be found between the skin of juveniles and adults. However, marked differences occurred between the skin structure of fry and juveniles. The modifications produced in the skin during the first weeks post-hatching are thus thought to contribute to an increase in the number of epidermal cells layers as well as a thickening of the dermis, producing the two strata commonly found in this area of the skin. The separation of the two dermal strata was not apparently present in the young fry.

Pickering and Richards (1980) described the epidermis of the alevin brown trout as having a concentration of goblet cells higher than at any other time during the life cycle. Small secreting cells were observed in the present studies in the epidermis of the rainbow trout fry, but they did not apparently correspond to an extremely high production of mucus.

The reason for this difference was explained by Blackstock and Pickering (1982) who noticed a very marked decrease in the number of mucous cells when the brown trout fry had reabsorbed the yolk sac and reached the free-swimming stage. They suggested that the high potential for production of mucus in the alevin stages was related to the need for protection against abrasion during the period of

residence in the gravel of the spawning redds.

In the juveniles and adults the differences in the upper and lower dermis were clearly established except in the head where the stratum spongiosum was much restricted. Roberts et al. (1970) referred to this layer as consisting of thin collagen fibres, arranged irregularly and acting as a matrix for numerous melanocytes. The differences found in the present studies in the dermal concentration of melanocytes is closely related to the normal pigmentation of the fish, explaining why the ventral skin sections had a lower concentration of such cells whenever compared with the head skin, where they were much more numerous.

Variations in epidermal thickness throughout the body were referred to by other authors (Roberts et al. 1970; Harris and Hunt, 1975 a). In the present studies the ventral skin showed a reduced number of layers of epidermal cells, especially in non- breeding fish. Furthermore, the staining characteristics of the mucous cells were not always the same. Pickering and Macey (1977), in their study on the structure and histochemistry of mucous cells in the char (Salvelinus alpinus), also found variations in the staining properties of mucous cells, which did not appear to be related to the state of maturity of the cells. Biochemical differences in the secretion of individual goblet cells from the same fish were thought to supply the answer.

The presence of melanin granules within the epidermis has not been frequently reported by authors who studied the normal salmonid epidermis. However, Percy (1970) described the presence of melanin granules in the epidermis of young goldfish (Carassius auratus) as well as macrophages heavily laden with pigment, which were thought to be moving outwards to the surface in goldfish kept in sodium chloride solutions. Melanin-containing macrophages in the epidermis were also described as conspicuous cellular elements in light microscopy sections of

the skin of plaice (Roberts et al. 1971). Their presence was also noticed in the epidermis of pigmented elvers and adults of Anguilla rostrata (Leonard and Summers, 1976). In previously reported work (Peleteiro, 1981; Peleteiro and Richards, 1985), melanin granules were occasionally seen in small aggregates, but only electron microscopical observations allowed the possibility of relating their presence to the different epidermal cells and structures (see 2.2).

The modifications observed in the skin of the mature fish during the spawning season reported here were similar to previous findings described by the present author, although the fish examined originated in quite different geographical areas.

The external changes which take place prior to spawning in the Pacific salmon (genus Onchorhynchus) were discussed by Smirnov (1959), who suggested a functional explanation for his findings. He described the structural transformation of the skin as maturity progressed, referring to the change in colouration and thickening of the integument. He also mentioned "absorption" of scales and an increase in the number of epidermal mucous cells, which would produce more slime. In 1960, Robertson and Wexler, in a study of histological changes in the organs and tissues of migrating and spawning Pacific salmon (genus Onchorhynchus) , reported the thickening process of the skin with an increased number of cell layers in the epidermis.

Stoklosowa, in 1966, also observed in skin sections of mature male and female sea trout (Salmo trutta trutta) a very thick dermal layer, but she described the male epidermis as formed by a single layer of small epithelial cells, in contrast to the females in which the epidermis was formed by many layers of distinct epithelial cells.It seems possible that Stoklosowa's unique observations on the male epidermis might be due to problems in the processing of the skin samples. The fact that a single layer of cells was found could be an artefact. In the present work loss of

epidermal layers was occasionally found in material that had not been fixed immediately after death.

In non-migratory lake trout (Salmo trutta , morpha lacustris), Stoklosowa (1970) observed that the dermis of the male was thicker than the female, but no differences were found in the epidermal structure.

In his study of the seasonal changes in the epidermis of hatchery-reared brown trout (Salmo trutta), Pickering (1977) showed the existence of significant sexual dimorphism in the skin structure of mature males and females, but such differences varied throughout the year. He divided the fish annual cycle into four periods: prespawning period (August-November); spawning period (December and January); postspawning period (February-April); interspawning period (May-July). It is interesting to note that such a chronological register is also applicable to the annual cycle of the rainbow trout reared in Portugal, which also spawn in December and January. He observed that the thickness of the brown trout epidermis fluctuated rhythmically during successive annual cycles, starting to increase at the prespawning period and reaching the maximum width at the end of the postspawning period. However, the epidermal thickness of the males was always significantly greater than that of the females. The number of mucous cells also fluctuated throughout the year, but the differences were more pronounced in the male, and especially decreased in December.

Richards (1979) also observed similar marked sexual dimorphism in lake brown trout (S. trutta). Temporal variations in the thickeness of the skin of the Atlantic salmon (Salmo salar) have been observed by Wilkins and Jancsar (1979).

Pickering and Richards (1980), in their study of the factors influencing the structure of the salmonid epidermis, gave an account of the changes occurring in the skin as sexual maturity proceeded, which will be compared with those of this

study in the following discussion.Changes in the skin structure of maturing male brown trout were recently described by Pottinger and Pickering (1985a), which included an increase in dermal and epidermal thickness, together with a reduction of superficial goblet cells.

The present findings of the changes occurring before, during and after the breeding season entirely agree with Pickering (1977), Pickering and Richards (1980) and Pottinger and Pickering (1985a), but are in disagreement with the descriptions of Stoklosowa (1966, 1970). A pronounced folding of the basement membrane was constantly found in mature males, accompanied by an increase in thickness of the epidermis and the dermis, where the scales could be noticed much more deeply buried than in the female epidermis.

Differences in the distribution of mucous cells did not always occur in the same manner as that described by Pickering (1977). However, in the later paper Pickering and Richards (1980) stated that although in mature and spent fish mucous cells were often reduced to zero, others showed increased numbers of these cells. In fact, in some of the males sampled during the breeding season in the present study the number of goblet cells was extremely high. The same contrast was noticed during previous studies by the present author (Peleteiro, 1981), in which a decrease in the number of such cells was noticed, more pronounced in the sea-water males than in males in fresh-water. Individual variations are certainly to be expected and in fact mucous cell concentration of individuals of the same species, sex and age was already found to be extremely variable (Pickering, 1974).

It is known that hormones, and especially androgens and oestrogens produce a hyperplastic response in the skin, when applied experimentally (McBride and Van Overbeeke, 1971; Yamazaki, 1972). Corticosteroids do not seem to have the same effect and, at present, there is no evidence of an elevation of their levels in sexually mature fish during the prespawning period (Pickering and Christie, 1981).

Discussing his results, Pickering (1977) admitted the implication of sex hormones in the sexual dimorphism he observed, because immature brown trout of similar size did not show the same changes. Furthermore 11-ketotestosterone was found to reproduce the epidermis-dermis rugae and the fall in mucous cell number seen in the mature brown trout (Richards, 1979 and Pottinger and Pickering, 1985 b). The mucous cell concentration has been reported by other authors as not to be under the control of sex hormones but possibly influenced by prolactin (Marshall, 1976).

Frequently observed in the present study at the light microscopy level were small cells with darkly staining nuclei, reported by Peleteiro (1981) and Peleteiro and Richards (1985) and previously referred to by some authors in the skin of salmonids (Roberts et al. 1970; Pickering and Macey, 1977; Richards, 1979; Pickering and Richards, 1980).

A significant epidermal infiltration with the same cell type was described as an occasional finding in the present observations on the skin of immature rainbow trout. This also suggests, perhaps not surprisingly, that although their number might be increased in spawning and spent fish epidermis, as some authors have described (Pickering and Richards, 1980), similar infiltrations may occur in immature fish with no relation to sexual maturation whatsoever. The electron microscopy observations, discussed later, helped in the identification of these cells and immunocytochemistry techniques shed some light on what their functional significance might be.

The epidermal lesions presently reported are similar to the skin changes described by Pickering and Richards (1980), who suggested a classification for the epidermal lesions found in mature salmonids. Such lesions were sometimes, but not exclusively, related to Saprolegnia infections (in many cases no fungal hyphae were observed). These authors also pointed out that the changes were regularly more pronounced in the males. In the present study these changes were found

predominantly in females, but, as more females than males were sampled, no firm conclusions could be drawn.

Lesions described by Richards (1979) and Pickering and Richards (1980) as type I, include a series of minor changes, such as the thickening of the skin of mature fish and the presumed-lymphocyte infiltration similar to that described in the present study.

Superficial cell sloughing was also reported in the present study which agrees with their description of type 2 lesions, namely "sloughing of areas of superficial epidermis is commonly found in association with such swollen surface cells". They also described type 3 lesions as large areas of epidermal sloughing, frequently not associated with fungal infection, with spongiosis or intercellular oedema in the surrounding areas. Severe spongiosis was also found in cases in the present study but not necessarily related to severe epidermal desquamation.

The Saprolegnia lesions observed in a female fish correspond to the type 4 and type 5 lesions described by Pickering and Richards (1980). In type 4 lesions extensive fungal infection occurred, spreading through the basement membrane into the dermal stratum spongiosum , with the development of bacterial infection. In type 5 lesions the superficial muscle was affected with fungal and bacterial penetration. An extensive inflammatory response was usually present as was noticed in the present case.

It was in a modified epidermis with severe spongiosis that the present study described the malphigian epidermal cells as having suffered extensive changes, appearing to be filled with eosinophilic granules. Although acidophilic granular cells have been described in the skin of certain salmonids, such as in the brown trout (Salmo trutta) by Blackstock and Pickering (1980), these authors were referring to a specific secretory cell type, which was found in the epidermis in low

numbers, totally different from the modified epidermal cells of this study In other teleost fishes, eosinophilic granular cells have also been described in the epidermis (Mittal et al. 1976; Phromsuthirak, 1977; Zaccone, 1979). But here again the descriptions did not agree with the present findings as was later confirmed by electron microscopical observation (see 2.2).

The present observations concerning the gill structure of juvenile and adult rainbow trout agree with the general descriptions of these organs made by several authors (Roberts, 1978; Yasutake and Wales, 1983). Roberts (1978) described the gill arch epithelium as a typical teleost epidermis, with large numbers of mucous cells.

Yasutake and Wales (1983) referred to the presence of numerous taste buds in the gill arch epithelium supplementing those scattered throughout the buccal cavity. Distinctive cellular aggregates were also found in the present study which closely resemble the skin taste buds of other teleost fishes described by Kapoor (1965), Hara (1971) and Bullock and Roberts (1974).

Morgan and Tovell (1973) reported that the gill secondary lamellae were covered by a double layer of epithelial cells, the outer one containing a large proportion of cells of several types including chloride cells, mucous cells and dark cells. These dark cells were described as unspecialized epithelial cells with microvillar folds at the surface and long lines of desmosomes attaching them to neighbouring cells. Intercellular spaces were formed between the two layers of epithelial cells, sometimes containing cellular elements closely resembling mammalian neutrophils (Morgan and Tovell, 1973).

The small cells with dark nuclei described in the epithelium of the gill arch and also in the primary and secondary lamellae, possibly represent lymphocytic cells, whose presence was noticed by Roberts (1978) below the gill arch epidermis and beneath the epithelial cells that cover the gill lamellae. However, they could also

represent the "dark cells" or the presumed neutrophils of Morgan and Tovell (1973). In fact, these latter authors, as well as Kendall and Dale (1979), did not describe lymphoid cells in their observations of the rainbow trout gill.

The structure of the thymus of the fry and juvenile rainbow trout described in this work entirely agrees with the descriptions of Grace and Manning (1980), Tatner and Manning (1982) and Chilmonczyk (1985). The thymus appears to develop just underneath the operculum from the 5th day before hatching, permanently remaining in a superficial position (Grace and Manning, 1980). The single cell layer epithelium that separates the organ from the gill cavity was found to be identical to the epithelium lining the pharyngeal cavity and therefore apparently not forming a specialized barrier (Tatner and Manning, 1982).

Chilmonczyk (1985) gave a detailed account of rainbow trout thymus structure, especially in juveniles and adults. He discribed septa of connective tissue, formed by foldings of the sub-thymic zone. According to this author the cross-sections of these septa presented a similar appearance to Hassal's corpuscles, which he claimed not to exist in trout thymus. He also reported that no thymic involution was seen in adult rainbow trout. Deanesley (1927) also referred to the absense of Hassal's corpuscles in the thymus of Salmo fario, but she described involution of the organ in both males and females, when the fish were between 2 and 2.5 years old. According to this author, involution was brought about by mass emigration of thymus cells and cessation of mitosis.

Deanesley (1927) described the presence of large cells with pale nuclei in the thymus of S. fario from three days before hatching and onwards, up to the adult stage. These cells were always referred to as mesodermal elements which would have migrated from the surrounding tissues. However, the only evidence to support this idea seemed to be the similarity between them and the cells of the underlying connective tissue, some of which were seen in close contact with the inner thymic

border. However, these cells also bear a close resemblance to the epithelial cells of the primitive pharyngeal epithelium, shown by the same author, before the differentiation of the thymic buds. These particular cells with large pale nuclei were also reported in the present work. The fact that they were always seen unrelated to the connective tissue septa suggests that they are independent from the mesodermal derived tissues.

Although there was considerable disagreement between earlier authors on the origin of the thymocytes, the studies of Deanesley (1927) and, much later, Grace and Manning (1980) seem to be quite unequivocal , showing that the thymus lymphoid cells originate by repeated cell division from the primitive epithelial cells that form the first thymic buds. The immigration of cells from other organs was not observed by these authors. They all reported that thymic cells were actually seen leaving the thymus. Deanesley (1927) who studied the histogenesis of the thymus in Salmo fario reported that migration of thymus cells occurred shortly after hatching, before the absorption of the yolk sac and during a period of rapid thymus growth, in a process not necessarily connected with organ involution.

Grace and Manning's (1980) work on rainbow trout added more information to Deanesley's (1927) observations. They found that the thymic cells were first seen outside the inner thymic capsule 15 days post-hatch, at the same time that small lymphocytes were first observed in the kidney. They thus suggested that such migrated cells might have contributed to the lymphoid cell population of the kidney. The migration of thymocytes to peripheral lymphoid organs in rainbow trout was subsequently traced by Tatner (1985) who labelled these cells in situ with tritiated thymidine. She was able to confirm that twice as many thymocytes migrated to the spleen as to the kidney.

The origin of the thymocytes in frog has been studied by Turpen, Volpe and Cohen (1973), who reciprocally transplanted the thymic primordia between diploid

and triploid chromosomally marked frog embryos. They showed that thymic lymphocytes originated directly from elements within the thymic primordium rather than from blood-borne stem cells which migrated into the organ. Virtually all lymphocytes in the spleen, kidneys and bone marrow of adult frogs were descendants of the original thymic stem-lymphocytes. Furthermore, they suggested that there was a self-perpetuating population of lymphoid stem cells in the intact thymus and that such a resident population is neither replenished nor replaced by immigrant stem cells from the bone marrow (Volpe and Turpen, 1985). The concept of the existence of stem-cells in the thymus could also apply to fish especially considering that which is presently known about the origin of the primitive thymocytes. The cells with large pale nuclei, already referred to in this discussion could be the equivalent to such stem cells of the frog thymus.

CHAPTER 2

ELECTRON MICROSCOPICAL OBSERVATIONS OF THE EPIDERMIS OF RAINBOW TROUT

This study on the ultrastructure of the epidermis of rainbow trout, follows previous observations, whose results have already been published (Peleteiro, 1981, Peleteiro and Richards, 1985).

However, more extensive studies have now been carried out in order to identify the presence of cells that, in the epidermis, may play a significant role in specific or nonspecific defense mechanisms.

Most fish used for light microscopy observations provided material for electron microscopy.

2.1 - MATERIALS AND METHODS

Adults - Two males (3+ and 5+) and four females (two 3+ and two 5+) obtained from MANTEIGAS, and three males and seven females (aged between 3+ and 4+) obtained from TRUTURÃO, were sampled for this study. The weights within the first group varied between 500 to 900 grammes and in the second between 400 to 750 grammes. Twelve juvenile rainbow trout, weighing between 70 and 200 grammes were also sampled.The material was collected from the dorsal, ventral and head skin in the adults, and lateral and head skin in the juveniles.

Immediately after removal from the fish, the fragments of skin were immersed in large drops of Karnovsky fixative (Karnovsky,1965), in which they were carefully cut into small fragments, approximately 1 mm^3, avoiding as much as possible, damage to the epidermis during this operation. Care in this preparatory phase was essential in order to produce good final results. Fixation was allowed to continue for

two hours at 4°C, followed by at least three washings of ten minutes in 0.1 M cacodylate buffer, pH 7.4. The material was then submitted to a second fixation in 1% osmium tetroxide (OsO_4) in 0.1 M cacodylate buffer, pH 7.4, for two hours at 4°C. Fixation finished, the fragments then underwent block staining with 1% uranyl acetate for one hour at 4°C. Dehydration in ethanol was followed by embedding via propylene oxide and a mixture of Epon and Araldite resin was used as embedding medium.

In order to select areas of epidermis for thin sectioning, semithin 2 µm sections were cut in an LKB Ultratome III, with a glass knife, and stained with 2.5% sodium carbonate and 1% toluidine blue. The same LKB ultratome was used to cut ultrathin sections, which were collected on uncoated copper grids. The sections were stained in 2% uranyl acetate (45 to 60 minutes at 60°C) and lead citrate (5-10 minutes), at room temperature.

Observations were made using a JEOL 100 C electron microscope, at 80 Kv (see appendix for details on the methods).

2.2 - OBSERVATIONS

The organization of the skin of the rainbow trout specimens was similar to that described for other salmonids, with the filament-containing cells (FCC) as the basic cell-type.

The epidermis was supported by a basement membrane (or basal lamina) of variable profile. In some cases only small indentations were formed (figure 26), whilst in others, a system of primary and secondary papillae could be seen (figure 27). The basement membrane complex was formed by a continuous dense layer (lamina densa), running parallel to the basal cell membranes, from which it was separated by a lighter layer (lamina rara). In the lamina rara very thin fibrillar elements could be seen crossing from the dense layer towards the basal FCC'S (figure 28). The membrane of these cells was more dense in the areas in contact with the basement membrane complex than in its lateral and distal portions adjacent to neighbouring cells.

The basal FCC's assumed a columnar appearance, with an elongated nucleus (figure 29). As they approached the superficial layers, both cells and nuclei showed a more polygonal shape. However, their ultrastructural appearance was similar at all levels of the epidermis (figures 30 and 31). One of their more conspicuous characteristics was the way these FCC's related to each other, through a very complex network of interdigitations of their cell membranes, and through desmosomes. The FCC's nuclei were usually large, often indented, with no obvious clumps of chromatin, which showed instead an homogenous distribution and a frequently prominent nucleolus. Most of the cellular organelles were located in the perinuclear area (figure 31) and internal structures such as small oval mitochondria, with poorly dense matrix, small smooth surface vacuoles and free ribosomes were seen. Occasionally, lysosome-like bodies could be seen, as well as elements of the Golgi complex. The peripheral area of the FCC's consisted of

regularly distributed bundles of tonofilament-like fibrils.

At the epidermal surface, the FCC's became very flattened with superficial microridges (figure 32) and a lighter cytoplasm. However, in some cases, the superficial FCC's kept their rounded profile, without microridges, assuming a necrotic appearance, with condensation of the nuclear chromatin and loss of cytoplasmic content, especially in the perinuclear area (figure 33).

Variable degrees of intercellular space formation were found in the epidermis of the mature male and female fish, more frequently than in the juveniles. In mild cases, the space was reduced, and the interdigitating folds of the FCC's cell membrane appeared in contrast to the low electron dense background (figure 34). In more severe cases the FCC's showed a rounded outline, losing most of their membrane folds, except in the vicinity of desmosomes (figure 35).

The other cellular components of the rainbow trout epidermis were the mucous cells, unicellular glands whose frequency was variable, as previously confirmed by the light microscopy observations. Those of the lower layers often appeared as immature cells, surrounded by FCC's, but with rare junctional complexes between them. At the initial stage of development, these cells showed the same size as adjacent FCC's, with a central nucleus of less dense chromatin and a large nucleolus (figure 36). The presence of abundant rough endoplasmic reticulum and a few vesicles containing flocculent material were suggestive of an initial secretion process.

In the more mature cells, the secretion vesicles occupied a larger volume, with a corresponding increase in cell size (figure 37). The electron density of the vesicles sometimes presented marked differences even within the same cell. The cell organelles and the nucleus were compressed against the base of the cell in a semi-lunar shape. As the mucous cells reached the surface, emerging between

FCC's, the mucous vesicles seemed to fuse to form larger ones (figure 38). Finally, superficial mucous cells were seen releasing their content into the surface (figure 39). Occasionally, pycnotic nuclei surrounded by rough endoplasmic reticulum could be seen at the surface of the epidermis, probably representing remains of spent mucous cells.

Lymphocyte-like cells were frequently located throughout the epidermis. A large dense nucleus, sometimes deeply clefted, with an equally dense nucleolus, surrounded by a narrow rim of cytoplasm, were distinguishing features of these cells (figures 40 and 41). A few mitochondria, with a matrix of medium electron density, free ribosomes, lysosome-like bodies and vesicles of variable diameter, were also regularly identified. No junctional complexes were observed in these cells. They were either surrounded by a light intercellular space, occasionally showing small pseudopodia (figure 42), or remained in contact with neighbouring cells (figure 41). These lymphocyte-like cells were present in most epidermal sections observed, both in adults and in juvenile fish. Their number was, however, quite variable. Especially in certain adults, cell aggregates were frequently noticed, and will be described later.

Macrophage-like cells were also observed, sometimes in higher numbers than lymphocyte-like cells. The nucleus was very variable in shape and the chromatin less electron dense than in the lymphocyte-like cells (figures 43 and 44). Their cytoplasm contained a variable number of lysosome-like particles, mitochondria of light dense matrix, Golgi complex, small amounts of rough endoplasmic reticulum and free ribosomes. No junctional complexes were observed in these cells.

In order to better understand how the last two types of cells described reached the epidermis, a careful observation of the basement membrane area was undertaken in an attempt to trace any crossing cells. It was in fact possible to locate, in a few sections, sites where the basement membrane was clearly

interrupted by a cell. Although it was difficult to identify with certainty the type of cells involved, the nuclear density and the cytoplasmic volume appeared to correspond to macrophage-like cells (figure 45).

The fact that only a few cases of the formerly described cells were found crossing the basement membrane is in contrast with the high number of such cells that were seen very close to it, in the epidermis. Both lymphocyte-like (figure 46) and macrophage-like cells (figure 28) were identified either isolated or in aggregates (figure 26). It is interesting to note that cells of the same type were also frequently seen in the dermis of the same skin sections close to the basement membrane.

Mention was earlier made that aggregates of lymphocyte-like and macrophage-like cells could be identified in the epidermis. Such groups of cells were found both in adults and in juvenile fish. Lymphocyte-like cells aggregates were, however, predominant in most sections observed (figure 47).

Although in many cases the cells in the clusters seemed to keep their oval to round shape, in others they assumed very irregular forms, as if compressed against each other and against the adjacent FCC's and mucous cells (figure 48). In this latter case, the nuclei of the cells often showed very irregular forms, sometimes apparently multilobulated (figure 49). The presence of mucous cells, either mature or immature, in close contact with the cell aggregates, was frequent, but not constant.

There was yet another cell type that was found in the epidermal sections of very few fish, whose ultrastructure does not agree with the cells already described. They were very frequent in the epidermis of a single adult female, but otherwise very seldom noticed. They usually showed a particularly clear cytoplasm, and a contrasting electron dense nucleus, in which deep clefts were present, often

making the cells look multinucleated (figures 50 and 51). The cytoplasm was filled with small electron dense granules (100-250 nm in diameter), sometimes separated from an enveloping membrane by a clear space or halo (figure 52). The other cell organelles consisted of a very few small mitochondria, small vesicles, a variable amount of rough endoplasmic reticulum and free ribosomes. Such cells usually showed an irregular outline and small portions of cytoplasm with the same ultrastructure could be seen in their vicinity, suggesting the existence of cytoplasmic extensions (figure 50). No junctional complexes were found between these cells and the surrounding ones. Cells with very similar ultrastructure were sometimes noticed in the dermis, underneath the basement membrane.

It was also in the ultrathin epidermal sections from the same female mentioned earlier, that large dense lysosome-like bodies, were found inside the FCC's (figure 53). At high power, their content looked finely fibrillar. Few of them contained small vesicles. In some areas the density of the vesicles was similar to the matrix of the lysosome-like bodies (figure 54). The cells displaying such dense bodies usually showed a decreased number of peripheral filament bundles. In epidermal sections of this very same fish as well as of other adult specimens large vacuoles of heterogeneous content of flocculent dense material and membranous formations were observed inside FCC's (figure 55).

The macrophage-like cells also displayed high numbers of heterogeneous bodies, with a content very similar to the previously described vacuoles of the FCC's (figure 56).

Cells with the ultrastructural characteristics of Merkel cells were not found in the epidermis. However, on rare occasions, interspersed between FCC's, certain cells were observed which shared some of the features of the above cited Merkel cells (figure 57). They were approximately the same size as the neighbouring FCC's, relating with them by desmosomes, but without forming membrane

interdigitations. The voluminous nucleus was moderately electron dense, with a prominent nucleolus. The cytoplasm was filled with filaments and sparse electron lucent vesicles.

Having described the ultrastructure of the epidermis, a brief account of the dermal area, just beneath the basement membrane follows. Bundles of collagen fibres and the melanin-containing cells were the most obvious features. The melanocytes formed a continuous bed of elongated cells, parallel to the skin surface. They showed long cytoplasmic processes, interspersed between collagen bundles, full of electron dense granules (figure 58). Microtubules could also be seen arranged in a longitudinal orientation in the cytoplasm (figure 59).Occasionally, electron lucent vesicles were also seen inside the cytoplasmic processes, with thin fibrils concentrically disposed, interspersed with small granules and some amorphous material (figure 60). Dermal macrophage-like cells also contained melanin granules inside what seemed to be lysosomal structures (figure 61). Such macrophage-like cells with apparently phagocytosed pigment were traced in the epidermis (figure 62). Eventually, melanin granules were found within FCC's in the basal epidermal layer (figure 63).

More than once, melanin-containing cells were seen moving into the epidermis through discontinuities of the basement membrane (figure 64). Some of these melanin-bearing cells, once inside the epidermis, assumed a severe modification of their nucleus, with a very irregular multilobulated aspect, with interspersed cytoplasmic processes between adjacent cells (figure 65).

The collagen fibres were arranged in regular bundles, orientated at different angles, so that they were observed in transverse or longitudinal planes. Fibroblasts appeared regularly as elongated cells between collagen lamella (figure 66), with a moderately dense nucleus, accompanying the shape of the cell. Occasionally, myelinated nerve fibres were seen (figure 67). Other cells typical of

this area of the skin were the iridophores. They showed their distinctive parallel arrays of plate-like clefts, surrounding a large nucleus of regularly distributed chromatin (figure 68).

Just as the dermis reached the lamina densa of the basement membrane, the regular pattern of collagen was replaced by a network of small reticulin fibres, which seemed to anchor into the continuous dense layer (figure 28).

In some dermal sections, usually presenting signs of cellular infiltration in the light microscopical observations, the organization of the tissue was disturbed by the presence of apparently inflammatory cells (figure 69). Whenever this occurred, the bundles of collagen showed signs of having been separated by the invasive cells, which could be seen at variable depths in the dermis.

2.3 - DISCUSSION

The ultrastructure of the skin of the rainbow trout specimens observed in the present study is in agreement with the results obtained in previous studies (Peleteiro and Richards. 1985) although a more detailed and complete scrutiny was made at present, mainly due to the higher number of fish sampled. The reported results also agree with the observations several authors have made on the teleost fish skin.

When reviewing the fixation methods for electron microscopical observations of the skin of fish, significant differences are found between the methods described by various authors.

Although paraformaldeyde, gluteraldehyde and osmium tetroxide are widely used, their concentration varies from study to study. Blackstock and Pickering (1980), in their ultrastructural studies on brown trout epidermis, experienced considerable difficulty in fixation and subsequent staining. From a variety of techniques tested they selected a procedure using 12% gluteraldehyde as first fixative, shortly followed by 1% osmium tetroxide as second fixative. This method was used in previous studies (Peleteiro and Richards, 1985) but the poor contrast on staining was not encouraging. Therefore, the widely used fixation method of Karnovsky (1965) was presently followed, with a block staining in 1% uranyl acetate which is believed to have improved the final results. The 2% aqueous solution of uranyl acetate was found to be very suitable for the final staining of the ultrathin sections, whenever this was carried out in the oven, at 50-60°C. At room temperature the sections had to be submitted to the same treatment for much longer periods with undesirable consequences, such as precipitate over the sections.

Most authors do not indicate the osmolarity of the buffer solution they have used, both in the preparation of the fixatives and for washing the tissue samples

after fixation. Some, however, indicate they have used 0.1 M cacodylate buffer (Hawkes, 1974a; Blackstock and Pickering, 1980) and others 0.1 M phosphate buffer (Leonard and Summers, 1976; Schwerdtfeger, 1978; Ferri, 1982). 0.1 M cacodylate buffer was used in the present work, but fixation problems were frequently encountered, such as cell retraction, loss of cell substance or increased intercellular space that were assumed to be artefacts, mainly due to the high osmolarity of the cacodylate buffer. The major problems were found in the sections of head epidermis which seems to suggest that the osmolarity of the buffer, at least for the treatment of head skin fragments should be lower than 0.1 M. Following on from the present experience, the best method for preparing samples of rainbow trout skin is likely to be a combination of the fixation technique of Blackstock and Pickering (1980) with a buffer of osmolarity less than to 0.1 M, together with a block staining with uranyl acetate to improve the final contrast of the thin sections.

Several studies have been carried out on the ultrastructure of the skin of salmonids such as Salmo irideus , by Jimbo, Shibukawa, Kobayashi, Soda and Kimura (1963); Atlantic salmon by Roberts et al.(1970): coho salmon, by Hawkes (1974 a,b); Atlantic salmon and brown trout by Harris and Hunt (1975 a,b) and brown trout by Blackstock and Pickering (1980).

All descriptions are unanimous in considering the filament-containing cells (FCC) and the mucous cells as the most numerous type of epidermal cells. However, the FCC'S are not described by all authors under this terminology. Hawkes (1974a) named them "keratinocytes", which is considered to be a misleading name for fishes, as only a very reduced number of species, certainly excluding salmonids, undergoes epidermal keratinization.

The designation of FCC's, which seems to have been initially chosen by Henrikson and Matoltsy (1968a), is currently used in most ultrastructural descriptions on fish epidermis, although some authors seem to avoid it, e.g. Lane

and Whitear (1977), Phromsuthirak (1977) or Mittal et al. (1980) who simply named them epithelial cells.

Wellings, Chuinard and Cooper (1967) and Brown and Wellings (1970), in their studies on the fine structure of the skin of the flathead sole (Hippoglossoides elassodon). referred to the FCC's as squamous cells, which does not seem to be a correct terminology. Although the epidermis in the telosts is a stratified epithelium, the only cells which could be named squamous are located in the superficial layers, assuming the characteristic flattened appearance. The FCC's in the basal layer can hardly be considered squamous cells.

In spite of the terminology disagreements, the observations of the various authors concerning the FCC's seem to agree with each other and with the present findings. The same is true of descriptions of the basement membrane (B.M.). Roberts et al. (1970), described the B.M. of the Atlantic salmon as an amorphous electron dense layer 600 to 800Å thick, separated from the epidermis by a light zone approximately 400Å wide. Harris and Hunt (1975 a) also referred to the same dense and lucent layers, whose thicknesses were 3 µm and 0,2 µm respectively. Although studying two different species of salmonids these latter authors did not refer to differences in the width of the B.M. between the species. However, their measurements do not seem to agree with those reported by Roberts et al.(1970), although they both studied the Atlantic salmon. Probably the B.M. layers do not present the same width throughout the different areas of the body, as was described by Phromsuthirak (1977) in the stickleback (Gasterosteus aculeatus). On the other hand the B.M. may suffer modifications which result in the differences in thickness observed. In fact, in the present observations, the B.M. was seen forming plicated systems of primary and secondary papillae, especially in the sexually mature males, whereas in others, it assumed an almost straight appearance. The shape and frequency of the infoldings of the B.M. among yearling coho salmon has been reported to vary considerably (Hawkes, 1974a).

Thin fibrillar elements were found in this study crossing from the B.M. dense layer towards the basal cell membranes. Such filaments have not been described in salmonids. Adepidermal granules have been found by Watanabe and Tachibana (1973) in the adepidermal space of Salmo irideus and dense granules with the same location were found by Hawkes (1974 a) in coho salmon, restricted to the embryo and not in the yearling. Leonard and Summers (1976) referred to the presence of fibrillar elements in the electron lucent adepidermal layer in the skin of the American eel (Anguilla rostrata), very similar to those reported here. Mittal et al. (1980) in Monopterus cuchia and Ferri (1982) in Pimelodus maculatus a fresh-water teleost, also reported the existance of fibrillar elements in exactly the same location.

The ultrastructural observations of the B.M. in mammals have shown that the electron dense layer is formed by a unique form of collagen, named type IV, as well as glycoproteins. This dense layer is called the basal lamina, and together with a mat of associated reticular fibres forms the basement membrane. The electron lucent layer separating the basal lamina from the basal surface of the epithelium should correspond to the thickness of the cell coat of the epithelial cells (Ham and Cormack, 1979).

The same structural organization seems to apply to the teleosts basement membrane. The thin filaments described in this study may be part of the cell coat of the epithelial cells, but only chemical investigations will show their exact composition and therefore disclose their origin.

A marked difference exists between the mammals and the salmonids in the attachment of the epithelium to the B.M., for no hemidesmosomes have been reported in the basal cells' surface (Roberts et al. 1970; Harris and Hunt, 1975 a). Such atttachment structures have, however,been described in other teleosts such as the American eel (Leonard and Summers, 1976) and the symbranchiform fish Monopterus cuchia (Mittal et al. 1980).

No matter how complex the B.M. system, the impression remains that it can be drastically modified, sometimes even to allow cell movement across it (see later in this chapter).

The various authors' accounts of the FCC's fine structure are very similar, not just for salmonids but for many other teleost fishes. They generally describe most cell organelles as close to the nucleus with the tonofilaments or intermediate filaments arranged in the external cytoplasmic area.

Most authors referred to the presence of filaments, without classifying them. Their size range varied from 60Å in the plaice (Roberts et al. 1971) to 80Å in the guppy, Poecilia reticulata (Schwerdtfeger, 1978). Others instead named them as tonofilaments e.g. Leonard and Summers (1976) in the FCC's of the American eel, Mittal et al. (1980) in Monopterus cuchia and Ferri (1982) in Pimelodus maculatus.

The most detailed description, however, came from the work of Harris and Hunt (1975 a) on the skin of Atlantic salmon and brown trout. These authors described the tonofilaments as 50-90Å wide and of variable length, forming a denser network in the more superficial cells than in the basal cells. They also observed a layer of finer filaments (30-50Å) immediately surrounding the perinuclear area, which they claimed to assume a banded appearence at very high power observation. Similar findings were reported by Henrikson and Matoltsy (1968a). They described in the FCC's of the guppy 70Å tonofibrils in the peripheral cytoplasmic area, as well as a loosely tangled network of lightly staining filaments between it and the perinuclear organelle complex. In both cases the above cited authors suggested these thinner (Harris and Hunt, 1975 a) or lighter (Henrikson and Matoltsy, 1968a) filaments could be the percursors of the peripheral tonofilaments.

Following the descriptions of Harris and Hunt (1975 a) the filaments observed in the rainbow trout FCC's fall into the category of tonofilaments, mainly due to their

size and similarity with the filaments found in the mammalian epidermal cells (Holtzman and Novikoff, 1984). However, a true understanding of the nature of the fish FCC's tonofilaments cannot be achieved without identification of their chemical composition.

Baden and Kubilus (1983) isolated fibrous proteins from the epidermis of carp (Cyprinus carpio), that had physical and chemical properties similar to the mammalian epidermal keratins.

Many authors referred to the existence of smooth surface vesicles as part of the organelles found in the FCC's (Wellings et al. 1967; Henrikson and Matoltsy, 1968 a; Bullock and Roberts, 1974; Harris and Hunt, 1975 a; Leonard and Summers, 1976). Identical vesicles were observed in the present studies as well as lysosomal-like bodies. Reports on lysosomal-like structures in the FCC's are rare. Schwerdtfeger (1978) described large electron dense vesicles which he presumed to be lysosomes in the FCC's of the guppy. He also reported the existence of vesicles produced in the dictyosomes, which would be electron lucent near the Golgi lamellae, becoming more electron dense at the surface of the cells. These vesicles were thought to contain the glycocalix material produced by the FCC's, which together with the mucous cells might contribute to the slime that covers the body surface. This same opinion was shared by Whitear (1970) who stated that in some bony fishes, the cuticle or superficial slime consisted of mucopolysaccharides secreted largely from the surface epidermal cells rather than from mucous cells. In the American eel Leonard and Summers (1976) reported the presence of a well-developed rough endoplasmic reticulum and Golgi apparatus, as well as numerous cytoplasmic vesicles within the intermediate and superficial FCC's, suggesting a certain degree of secretory capacity in addition to their structural role.

Histochemical studies involving the epidermal cells of Amphipnous cuchia (Hamilton), a symbranchiform fish, were undertaken by Banerjee, Agarwal, Rai

and Mittal (1976). The absence of acid phosphatase activity and the presence of strong succinic dehydrogenase activity in the superficial epidermal cells, even in the outermost layers, led these authors to suggest that these cells would remain metabolically active. The idea of secreting epidermal cells was again put forward by Whitear and Mittal (1984), who showed the presence within these cells of electron lucent or moderately lucent vesicles, whose content was possibly sulphate acid glycoproteins identified by histochemical methods.

The changes observed in the FCC's as they approach the skin surface have been described in the present work. Some of them were probably undergoing necrosis, the images of their nucleus corresponded to the descriptions of Ghadially (1982) on nuclear changes occuring in necrotic cells. According to this author, pycnosis involves a shrinkage of the nucleus and condensation of chromation and in karyorrhexis the nuclear chromatin is aggregated into numerous masses, later released by rupture of the nuclear envelope. More seldom, cells in karyolysis have been observed, with the nuclear envelope remaining intact, but the contents partially or completely lost. In previous work of the present author, cells showing karyolysis have also been found in the epidermis of rainbow trout (Peleteiro and Richards, 1985).

The signs of cell necrosis in the superficial layers, however, were not restricted to the nuclear changes. A marked loss of cytoplasmic contents was noticeable, as was described by Roberts et al. (1970) and Harris and Hunt (1975 a). The first mentioned authors also reported that the morphology of the superficial cells varied from block to block, in the same fish, depending on whether the outermost cells were about to slough or not. From the present observations it seems likely that in those cases in which the superficial cells, including the penultimate layer, were not assuming the typical flattened shape, this represented increased epidermal sloughing. In such cases even the stretched desmosomes between the cells of the two superficial layers, described by some authors as providing the basis for the

elongated superficial ridges (Bullock and Roberts, 1974),were missing. These ridges form an intricate pattern demonstrated by scanning electron microscopy by Hawkes (1974 a) in the coho salmon and described as long microvillar ridges in the Atlantic salmon by Harris and Hunt (1975 a). It seems that the microridge pattern of the epidermal cells of teleosts can be modified when sodium chloride is added to the aqueous environment (Ferri, 1983 b), but in the present study the fish were always kept in fully fresh-water.

The increased intercellular spaces occasionally found in the epidermal sections observed in the present study were not reported as a common finding by other authors who studied the skin of salmonids. However, similar aspects were evident in electron micrographs of trout head epidermis published by Harris and Hunt (1975 a).

Schwerdtfeger (1978) referred to the basal FCC's in the epidermis of the guppy as often separated by wide intercellular spaces. As was mentioned earlier, fixation problems were detected in the course of the present work. It is therefore almost impossible to differentiate between true intercellular spaces and what may really be artefact. However, marked intercellular spaces were found in a few sections between cells of the basal layer, which were closely adjacent to other cells, demonstrating that these spaces were real and not artefact. Such spaces could be the electron microscope image of the spongiosis, often noticed in light microscopical section.

The mucous cells identified in the present work in the epidermis of rainbow trout were similar in every respect to the same cell type described by other authors in salmonids (Roberts et al. 1970; Hawkes, 1974 a; Harris and Hunt, 1975 b). In fact,the various authors' accounts of this cell type are quite similar for many teleost species (Henriksen and Matoltsy,1968 b; Brown and Wellings, 1970; Roberts et al. 1971; Leonard and Summers, 1976; Schwerdtfeger, 1978; Mittal et al. 1980). The reports on

the ultrastructure of immature mucous cells are again identical, except for Roberts et al. (1970) who did not appear to find any mucous cells at this stage of development, for they reported that the earliest recognizable mucous cell seen in the head epidermis of the Atlantic salmon resembled the mature cells.

In the course of the present study, the immature mucous cells have never been found close to the basement membrane, but rather above the basal layer. Their most conspicuous feature was in fact the extremely abundant rough endoplasmic reticulum, arranged around the nucleus, together with smooth surface vesicles aggregated to one side of the nucleus and full of the flocculent material also present in the more mature cells, which is the actual mucous secretion.

Henriksen and Matoltsy (1968 b), in their observations of the mucous epidermal cells of three teleost species, reported the presence of vesicles on the convex surface of the Golgi complex in contrast to vesicles containing mucus which were accumulating in the concave surface. They consequently suggested the existence of a unidirectional transport in the production of mucin. The same is known to occur in the intestinal goblet cells (Berkaloff, Bourguet, Favard, Lacroix, 1978): the proteins synthetized in the rough endoplasmic reticulum are transferred to the Golgi apparatus, where they suffer a glycosylation process by addition of the carbohydrate component of the glycoprotein secretion. Secretory vesicles finally leave the Golgi apparatus through the mature or concave surface

The goblet cells of the fish epidermis show a very marked ultrastructural resemblance to the goblet cells of the mammalian pseudostratified respiratory epithelium or of the intestinal epithelium. It seems therefore likely that in most functional aspects they might show similar characteristics. However, the way in which the secretion product is extruded from the cells does not seem to be identical.

In the mammalian goblet cells, the secretion vesicles bud off from the

uppermost Golgi saccule and move to the cell membrane, where their membranous walls fuse with the plasmalemma and their content is emptied to the exterior of the cell, in a process known as exocytosis (Ham and Cormack, 1979). This process, typical of merocrine glands, occurs in such a way as to keep the cell membrane intact, so that there is no loss of cytoplasm. This secretory process seems to be continuous or at least cyclical, in the respiratory mucous membranes, so that when the cells empty their contents they develop regularly arranged microvilli on their free surface. Such cells have been termed brush cells (Ham and Cormack, 1979).

The present findings are similar to those of other authors in that the goblet cells in fish achieved the mature stage long before reaching the surface. On occasion, superficial mucous cells were found with empty cell membranes, with the nucleus and still numerous cisternae of rough endoplasmic reticulum present. These observations suggest that once their content had been released, these cells did not go on producing more mucus, contrary to the situation in mammals. Their remains possibly became part of the superficial layer of the skin, where necrotic cells were frequently found.

The process by which the mucus is formed at the skin surface was also reported by Roberts et al. (1970), Hawkes (1974 a) and Harris and Hunt (1975 b). They unanimously described the mucous cells which had progressed to the level of the surface, as releasing their membrane bound mucus by dehiscence of the cell membrane. They also reported that, occasionally, three or four intact vesicles were seen at the outer surface.

Another statement, common to various authors, was the difference in electron density showed by the mucous vesicles, even within the same cell (Roberts et al. 1971; Bullock and Roberts, 1974; Harris and Hunt, 1975 b; Whitear and Mittal, 1984). Differences in vesicle density and shape were also reported by Roberts et al.(1970) in the epidermis of the Atlantic salmon. These differences in electron opacities of

the content of the mucous vesicles may reflect differences in their carbohydrate and protein content as was suggested by Harris, Watson and Hunt (1973) and Harris and Hunt (1975 b).

Some authors identified desmosomes between the mucous cells and the FCC's, although never so numerous as between contiguous FCC's (Henrikson and Matoltsy, 1968 b; Brown and Wellings, 1970; Harris and Hunt, 1975 b). Desmosomes between mucous cells and FCC's have been reported in the present study but goblet cells showing no junctional complexes were also common, probably due to the plane of the section. The presence of such complexes is of importance when considering the origin of the mucous cells in the fish epidermis, although desmosomes in general are far from being exclusive of the epithelial cells.

In mammals, it is believed that goblet and ciliated cells of the pseudostratified epithelium are both derived from basal stem cells, which divide and differentiate to take the place of those that are lost (Ham and Cormack, 1979). The same mechanism apparently takes place in the epithelium lining the intestine. The stem cells in the crypts divide and differentiate to replace the lost ones (Ham and Cormack, 1979). This suggests that the epidermal mucous cells also probably originate from basal epidermal cells, initially FCC's, which develop an abundant rough endoplasmic reticulum and Golgi complex, never completely losing the filaments. In fact, filaments were regularly observed during the present work within mucous cells and other authors have also reported their presence (Henrikson and Matoltsy, 1968 b). As a consequence of their study of the lamprey epidermis, Downing and Novales (1971) suggested that undifferentiated epidermal cells retained the potential to develop to FCC'S or mucous cells.

The presence of lymphocyte-like cells in the epidermis of rainbow trout has already been well documented in previous work by the present author (Peleteiro, 1981; Peleteiro and Richards, 1985). As was then reported, several authors have

referred to the presence of cells with similar characteristics to the ones described as lymphocyte-like cells in ultrastructural studies of the epidermis of teleost fishes (Leonard and Summers, 1976; Phromsuthirak, 1977; Ferri, 1983 a). The cells, named as lymphocytes by the above authors, had the same simple ultrastructural organization described for the lymphocyte-like cells in the present work. The commonly described features were a very dense nucleus, occupying most of the cell volume, surrounded by a narrow rim of cytoplasm with a paucity of intracellular inclusions. Other authors have reported the presence of lymphocytes in their electron microscopical observations of the epidermis of fish, without giving a detailed description, or showing electron micrographs of such cells (Brown and Wellings, 1970; Lane and Whitear, 1977; Mittal et al. 1980).

Several ultrastructural studies on the haemopoietic organs of chondrostean elasmobranchs and teleost fishes have reported the organization of the lymphocytes in the thymus, the spleen or the kidney of these groups of fish (Clawson, Finstad and Good, 1966; Smith, Wivel and Potter, 1970; Zapata, 1979; Pulsford, Fange and Morrow, 1982; Tatner and Manning, 1982; Pulsford, Morrow and Fange, 1984). Studies on blood leucocytes of teleosts (Weinreb, 1963; Ferguson, 1976; Cannon, Mollenhauer, Eurell, Lewis, Cannon and Tompkins, 1980) and elasmobranchs (Morrow and Pulsford, 1980) also referred to the ultrastructural organization of the blood lymphocytes. Small pseudopodia, as mentioned in the present study, were regularly reported by several authors as a distinctive feature of these cells. Differences in size between lymphoid cells seem to be common not just in the peripheral blood but also in the haemopoietic organs. The largest diameter reported, 8.2 µm, was found in the study of Weinreb (1963) on the blood cells of Carassius auratus, and the minimum size, 2.0 µm, in the thymus of the dogfish (Pulsford et al. 1984).

The cells described in this study as macrophage-like, with a moderately dense nucleus, variable amounts of lysosome-like particles, and large heterogenous

vesicles, had similar ultrastructural characteristics to the mammalian macrophages. The fine structure of these cells was described as irregularly outlined cells because of numerous pseudopodia, surface folds and finger-like processes projecting from them in various directions. Furthermore, macrophages in mammals contain several types of lysosomes, including phagosomes, membrane bound vesicles with an heterogenous content of phagocytosed material (Ham and Cormack, 1979).

Phromsuthirak (1977) also described macrophages in the stickleback epidermis, which he recognized by the large phagosomes. He also described membrane bound granules and vesicles, which are similar to the lysosome-like structures presently described inside the epidermal macrophage-like cells. This same author studied the changes produced during wound healing. He found that two days post-wounding there was a very marked increase in the number of macrophages in the epidermis, which accumulated between the layers of epithelial cells, migrating from both sides of the wound. On the contrary, the number of lymphocytes did not significantly increase during the healing process. This concept of macrophages migrating from the dermis towards the surface had already been put forward by Bullock and Roberts (1974), who described the presence of macrophages in the epidermis of marine teleost fish, which might have gathered effete melanosomes from the dermal stratum spongiosum.

"Crossing-cells", moving through the basement membrane,were described in the present work. The presence of numerous membrane-bound vesicles and the moderately dense nucleus tend to identify these crossing cells as macrophages. Although there was a large number of macrophages and even lymphocytes seen close to the epidermal side of the basement membrane, and assuming that at least the macrophages were intrusive cells, it seems likely that the crossing process is very quick as it is so rarely seen actually taking place.

Great difficulty was encountered in classifying the apparently multinucleated cells, so frequently found in the epidermis of both adults and juvenile fish. The density of the nuclear chromatin seemed to indicate that they were macrophages, whose extremely indented nucleus would be responsible for the multiple nuclear profiles. However, in a few cases, the total volume of these cells and the space between some of the nuclear material made if difficult to accept that they could represent a normal mononucleated cell. It is possible, of course, that they were multinucleated cells. Giant cell formation has largely been described in inflammatory responses in fish (Carmichael, 1966; Timur, Roberts and McQueen, 1977; Richards, Holliman and Helgason, 1978). However, according to the literature, the fine structure of giant cells in human sarcoid nodules (Ghadially, 1982) or in subcutaneous granulomas (van der Rhee, van der Burgh-de Winter and Deams, 1979) was quite different from the apparently multinucleated cells previously described.

References to the ultrastructure of the fish giant inflammatory cells have not been found by the present author. However, as in light microscopical observations they show similar features to the mammalian cells, their fine characteristics should also be identical.

Cells showing a particularly clear cytoplasm, filled with small electron dense granules and vesicles, were described in the present study as frequent in the epidermis of a female fish.

Ferguson (1976), describing the ultrastructure of plaice (Pleuronectes platessa) leucocytes, gave the following account of the monocytes: irregular outline, electron lucent nucleus, apart from a narrow peripheral band of chromatin; large ovoid cytoplasm with round mitochondria, rough endoplasmic reticulum and a centrosomal area. He also reported the existence of small primary vesicles, which could be seen forming from Golgi stacks. The vesicles were of varying size and

electron density, the smaller ones being more electron lucent.

In an ultrastructural study of the leucocytes of the channel catfish (Ictalurus punctatus) by Cannon et al.(1980), the monocytes showed similar features to the plaice homologous cells: condensed chromatin, often located adjacent to the nuclear envelope, abundant rough endoplasmic reticulum, large rounded mitochondria, dictyosomes and granules. These granules could be seen in the electron micrographs,shown as moderately electron dense with a clear halo separating them from the limiting membrane. Cannon et al. (1980) described these granules as lacking internal structure, in contrast to the granules of other leucocytes,which showed a crystalline or striated structure in their central part.

The monocytes in the peripheral blood of the dogfish (Scyliorhinus canicula) were briefly described by Morrow and Pulsford (1980), who reported numerous fine pseudopodia, pale cytoplasm with mitochondria, Golgi profiles and small electron dense granules (diameter 150 to 300 µm). These authors claimed that they often found clumps of glycogen in the monocytes. Cannon et al. (1980) also referred to the presence of glycogen in monocytes, but in much lower quantities and never in clumps.

The mammalian monocytes described by van der Rhee et al. (1979) were said to have numerous, small, round, oval or elongated electron dense granules (100 - 450 µm in diameter) limited by a single membrane. They also found a clear halo separating the contents from the limiting membrane, in some of those round granules.

Nichols, Bainton and Farquhar (1971) studied granule production in monocytes of various species, indicating that two populations of granules seemed to be present, although they would be morphologically indistinguishable. A first type (primary granules) formed during the promonocyte stage in the bone marrow, which could

be regarded as primary lysosomes due to their enzymatic content and secondary granules produced in the bone narrow monocytes and in the circulating monocytes and whose content was not possible to identify.

Most of the characteristics found in the monocytes by these authors apply to the above referred to electron lucent cells especially concerning the cell profile, the shape and density of the nucleus, the centriole, the numerous granules and vesicles and the pale cytoplasm. Cytoplasmic particles suggesting the existence of glycogen deposits have not been found. However, this does not preclude their presence prior to fixation, for it is known that the preservation of glycogen is largely dependent on the processing method used (Vye and Fischman, 1970, 1971).

According to the observations reported in the present thesis lysosome-like bodies were frequently noticed in the cytoplasm of the FCC's. Novikoff (1961) described the ultrastructure of lysosomes stating that their only morphological characteristic was the single unit enveloping membrane. This same author later claimed that the presumptive identification of cytoplasmic particles as lysosomes was only possible if they were bound by a membrane and if cytochemical studies showed that they possessed at least one of the hydrolytic activities identified in lysosomes through biochemical studies (Holtzman and Novikoff, 1984).

No attempts have been made in the present work to identify hydrolytic enzymes in the lysosome-like bodies; therefore, no firm conclusions can be drawn from the reported observations. In spite of this, the large electron dense vesicles found inside the FCC's seem to share many of the lysosome ultrastructural characteristics.

In the present thesis, electron micrographs were shown in which it could be seen that some of the lysosome-like bodies in the FCC's had small vesicles with a matrix of variable density. These structures show a close resemblance with multivesicular bodies, described by Ghadially (1982) as vacuoles containing vesicles

set in a lucent or dense matrix. This author also stated that,since acid phosphatase had been demonstrated in multivesicular bodies from various sites, they were considered to be a variety of lysosomes. However, considerable controversy still exists about their origin, as Ghadially also explained in a brief literature review on this matter (Ghadially 1982). The various ideas mentioned relate the multivesicular bodies to primary lysosomes (Novikoff, Essner and Quintana, 1964, cited by Ghadially, 1982), to Golgi vesicles (Friend, 1969 cited by Ghadially, 1982) or even to autophagic vacuoles in which several vesicles would have been enclosed (Ericsson, 1964, Kessel, 1966, both cited by Ghadially, 1982).

Assuming that large electron dense single membrane bounded structures observed in the present study were in fact lysosomes, the problem still remains of explaining why and how they formed in the FCC's,for it does not seem possible to consider them as normal structures.

These large electron dense bodies correspond to the eosinophilic granules observed in the epidermal cells of a female fish, reported in chapter one of the present thesis (see 1.2). The epidermis of this female was undergoing a degenerative process, with marked spongiosis. The electron microscopical observations helped to confirm the changes in this epidermis, showing large intercellular spaces and a rounded profile of the FCC's even in the basal layer. It was pointed out that the cells with the electron dense granules seemed to have fewer filaments in their cytoplasm than usually found in FCC's. This observation suggests that an autophagic process could be taking place within these cells, and the presence of very thin filaments inside the dense bodies could represent the remains of the cell filaments. However, the absence of cytochemical evidence for acid phosphatase inside these structures prevents their more certain identification as lysosomes.

This same problem was encountered whenever attempting to classify with certainty the heterogenous bodies which were also found inside some FCC's, as well

as in macrophage-like cells. Sometimes their content could be related to membranous structures suggesting that reticulum cisternae. Golgi stacks or isolated vesicles could have been involved in an autophagocytic process. However, for most of these heterogenous bodies it was not possible to identify their content.

It is interesting to note that in the FCC's, where such lysosome-like bodies were found, the Golgi complex was regularly much more developed than usual, as if the secretory function of the cell had suffered increased demands. As Ghadially (1982) pointed out, distinction in practice between phagolysosomes (exogenous content) and cytolysosomes or autophagic vacuoles may be simple, difficult or impossible. For this author the cytolysosome is characterized chiefly by sequestrated cell organelles and cytomembranes within its substance, yet such membranes may at times be exogenously derived by phagocytosis of a fragment of another cell.

The same type of heterogenous structures were found inside presumed macrophages in the course of the present study. Their relation to secondary lysosomes does not seem controversial, in spite of the lack of enzyme identification. Macrophages are known to be highly phagocytic and the presence of ingested material surrounded by a membrane may represent the presence of phagosomes, in which ingested material would be submitted to the action of the enzymes of primary lysosomes when the membranes of both structures fuse, forming phagolysosomes (Holtzman and Novikoff, 1984).

As for the light microscopical observations, no cells with the characteristics of the acidophilic granular cells described by Blacksotck and Pickering (1980) in the skin of brown trout,were found in the thin sections of the rainbow trout epidermis. Similarly, no sacciform cells were found like the ones described by Pickering and Fletcher (1986) in the epidermis of brown trout and char.

As was stated in the observations no obvious Merkel cells were found in the skin

of rainbow trout. However, a description of a particular cell type was provided which agrees in many details with the report made by Lane and Whitear (1977) of the Merkel cells in the epidermis of Ictalurus melas and Phoxinus phoxinus.

The cells observed in the present work and those described by the above authors had a large nucleus, pale cytoplasm with filaments and desmosomes. The Merkel cells observed by Lane and Whitear (1977) in the epidermis of Ictalurus melas had intracytoplasmic vesicles, some of them clustered near synapses. The cells observed in the rainbow trout epidermis also had vesicles, but they did not seem to be associated with any particular internal or external structure. Peripheral processes were not seen in these cells, but Lane and Whitear (1977) observed that several serial sections had to be examined to identify such processes in the fish Merkel cells and also to disclose adjacent nerve fibres.

Merkel cells, or their equivalent, possibly exist in the epidermis of rainbow trout. However, their distribution may not be uniform, for it is known that Merkel cells in mammals tend to be associated with richly innervated areas (Bloom and Fawcett, 1975).

The dermal fine structure of teleosts has also been extensively studied and the collagen fibres arrangement and cells described in the present study agree with most other authors' observations (Brown and Wellings, 1970; Roberts et al. 1970, 1971; Bullock and Roberts, 1974; Hawkes, 1974 a).

In species such as the American eel, the orientation of the dermal strata is the opposite of that observed in most species studied, including the salmonids (Leonard and Summers, 1976). The same reversed organization of the dermis was described by Ferri (1982) in the teleost Pimelodus maculatus. In spite of this, the cells and fibres described in these cases were similar to what other authors found in most teleosts studied.

Interesting observations were made in the present study concerning the melanin production in the dermis. A large number of melanin-containing cells was detected which closely resemble the cells described by most authors as melanophores (Brown and Wellings, 1970; Roberts et al. 1970, 1971; Hawkes, 1974 b) or melanocytes (Leonard and Summers, 1976; Ferri, 1982) in the dermis of several teleost fish, including salmonids (Roberts et al. 1970; Hawkes, 1974 b). Hawkes(1974b) considered the melanin-containing cells, which he named melanophores, as part of a chromatophore unit, which included xanthophores and iridophores, in the dermis of coho salmon. This author found a bimodal size distribution of these cells in the fingerling and in the smolting yearling salmon, which he claimed not to have found in the non-smolting yearling fish.

In mammals, melanin producing cells have been named melanocytes (Ham and Cormack, 1979) and are found beneath or between the cells of the basal layer of the epidermis. It is also known that these are the cells that contain tyrosinase, the enzyme essential in the synthesis of melanin, and therefore the only positive cells to the DOPA (3,4 dihydroxiphenylalanine) reaction (Block, 1927, cited by Ghadially, 1982; Fitzpatrick, Becker, Lerner and Montgomery, 1950).

At the Sixth International Pigment Cell Conference held in 1965 (Fitzpatrick, Quevedo, Levene, McGovern, Mishima and Oettle, 1966) the melanocyte was defined as a cell which synthesizes a specialized melanin-containing organelle. These organelles, the melanosomes, were studied and classified in four stages (Fitzpatrick et al. 1971, cited by Ghadially, 1982). Stage I melanosomes are small vesicles, derived from the Golgi complex, containing tyrosinase previously synthesized in the rough endoplasmic reticulum. In stage II melanosomes a complex internal structure can be noticed, but melanin is not yet present. These vesicles are also known as pre-melanosomes. Deposition of melanin on these structures characterizes the stage III melanosomes and the completion of the process produces electron dense granules, which prevent the identification of the internal structure (stage IV melanosomes).

At the above cited conference a special reference was made to the melanocytes of cold-blooded vertebrates that form an integral part of the mechanism of colour change by intracellular displacement of melanosomes. These cells were named melanophores.

In the present study, melanocytes were described as containing electron dense granules and occasionally, vesicles with a fibrillar content, interspersed with small granules and some amorphous material. The electron dense granules appear to be stage IV melanosomes and the vesicles stage II melanosomes or premelanosomes. Information concerning the presence of electron lucent vesicles in fish dermal melanocytes was very scarce. Roberts et al. (1971) found electron lucent vesicles of amorphous material in cytoplasmic processes of dermal melanocytes in plaice. They interpreted their findings as processes of melanophores from which melanin granules had been withdrawn. According to the electron micrographs shown it seems more likely that they were in fact stage I or stage II melanosomes.

Brown and Wellings (1970) found a cell which they described as an immature melanophore in the epidermis of Hipoglossoides elassodon . They reported the presence of incompletely melanized granules with internal rod-like subunits. A careful observation of these structures showed that they do not resemble melanosomes at any stage of development. The electron micrograph shows, in fact, what appeared to be a leucocyte, with moderately electron dense lysosome-like bodies, separated from the surrounding membrane by a clear halo. The characteristics of these bodies, with internal rod-like fibrils, seem to correspond to the specific granules described in the neutrophils of plaice (Ferguson, 1976) or the neutrophils of catfish (Cannon et al. 1980). Ferri and Macha (1982) also described cells with similar fine structure in the epidermis of Pimelodus maculatus, which they named heterophils.

Stage III melanosomes were the only type of pigment granule reported by

Hawkes (1982) in melanophores of albinistic rainbow trout. This author described them as membrane-bound organelles, 0.5 µm in diameter, partially melanized with a fibrillar structure .

Apart from melanosomes, another structure is very characteristic of the melanocytes: the cytoplasmic microtubules described by most authors (Roberts et al. 1970, 1971; Hawkes, 1974 b; Leonard and Summers, 1976). These microtubules seem to be responsible for the movement of the pigment granules within the melanocytes, either concentrating them in a small area in the cell, or dispersing them into the cytoplasmic processes (Schliwa and Bereiter-Hahn, 1973). Murphy and Grasser (1984), studying the cytoskeleton of melanophores from two species of marine fish, concluded that the filaments were different from actin and tubulin and they could entrap the pigment granules coordinating their dispersion within the cytoplasm.

The role played by these microtubules, concentrating and dispersing pigment, may have some influence in the difference in size found by some authors for the melanin-producing cells (Brown and Wellings, 1970; Roberts et al. 1971; Hawkes, 1974 b), because the variations noticed were mainly due to the size of the pigment--laden cell processes

Not many recent references were found in the literature reporting the results of the DOPA reaction in sections of fish skin. In his review work on fish chromatophores and pigments, Fujii (1969) referred to several authors who identified tyrosinase in the skin of teleost fish. It is considered that the molecules of the enzyme tyrosinase do not have the same physical and chemical characteristics in different species. Roberts et al. (1971), in their work on the structure of the skin of plaice, failed to detect any positive DOPA reaction.

Other types of melanin-bearing cells were seen in the dermis of the rainbow trout examined in the present study, both in mature and immature fish. They were

named dermal macrophage-like cells and seem to represent the same cell type described in mammals as melanophages, dermal cells which contain phagocytosed melanin (Ghadially, 1982). In fact, these cells, apart from the usual characteristics of macrophages, also showed electron dense granules inside single membrane bound structures, frequently more than one granule, together with some moderately dense material. These structures appeared to be the equivalent of the melanosome complexes of the melanophages in which Hori, Toda, Pathak, Clark and Fitzpatrick (1968) demonstrated acid phosphatase activity, therefore being regarded as lysosomes.

The terminology applied here for these macrophage cells containing melanin is debatable. Again, at the Sixth International Pigment Cell Conference, the term melanophage was deleted from the terminology proposed for vertebrate melanin-containing cells. Macrophages were regarded then as incidentally pigmented cells.

The melanin-containing cells that have been found crossing the basement membrane during the present study always seemed to be melanin-bearing macrophages. This was mainly due to the presence of the above described melanosome complexes in their cytoplasm, which together with other characteristics, such as nuclear density and absence of microtubules, supports this hypothesis. Such cells were also seen in between the epidermal layers, with large melanosome complexes, where several melanin granules were mixing with other phagocytosed material. The melanin-bearing cells that were described in the present thesis as assuming a very irregular nuclear shape and sometimes appearing multinucleated, could be melanin-bearing macrophages undergoing degenerative changes and releasing their melanin granules.

In the present study, melanin granules were found within FCC's either as isolated granules, or inside what appeared to be heterolysosomes, together with

some other material of membranous or fibrillar structure. The granules could possibly be taken up by the FCC's by some process identical to that which occurs in mammalian keratinocytes (Wolff and Hönigsmann, 1971). Phromsuthirak (1977) described the epidermal cells of the stickleback as phagocytic and the basal cells seemed to be particularly active, especially when phagocytosis was confirmed by experiments with carbon particles and with horseradish peroxidase. Yoshizaki and Tamura (1979) also reported the presence of melanin granules in the inner layer epidermal cells of a mormyrid fish (Gnathonemus petersii).

The traffic of melanin-bearing macrophages corroborates in some way our previously described observations of macrophage-like cells crossing the basement membrane. In fact, melanophages moving towards the epidermis were more frequently seen than "empty" macrophages.

The traffic of macrophages from the dermis to the epidermis has been previously described by Percy (1970) in the goldfish. This author thought that newly formed dermal melanophores would degenerate and become engulfed by macrophages,which would then move outwards to the epidermal surface. Melanin--containing macrophages were also very prominent within the epithelium of the dorsal surface of plaice, as described by Roberts et al. (1971). These authors found such macrophages at the superficial epidermal layer, the melanin granules being voided at the surface in the same dehiscent fashion as the mucus. Although several melanin-bearing macrophages were identified in the rainbow trout skin, images such as the ones showed by Roberts et al. (1971) were never found.

Macrophages laden with melanin granules, probably engulfed in the sequence of damage to the skin, were seen migrating through the epidermis of healing lesions in salmon parr (Roberts, McQueen,Shearer and Young, 1973). In their review of the skin structure of marine teleosts, Bullock and Roberts (1974) referred to the presence of macrophages in the epidermis,which would have gathered effete

melanosomes from the dermis.

Leonard and Summers (1976), describing the skin of the American eel, reported having found melanocytes both in the dermis and epidermis. No electron micrographs of the epidermal melanocytes were shown, but the authors reported that they had the same ultrastructure as those in the dermis. Melanin-bearing macrophages can be found in many internal organs in teleosts, such as the spleen, kidney and liver (Roberts, 1975) and their functional significance has been recently reviewd by Agius (1985).

The cells described in the present study as iridophores showed a very close resemblance to the cells described under this heading by various authors in the dermis of teleost fishes (Brown and Wellings, 1970; Bullock and Roberts, 1974; Ferri, 1982). The iridophores seem to be reflecting cells, responsible for the silvery appearance of the sides of the fish (Denton and Nicol, 1966). In their cytoplasm, purines (predominantly guanine) exist as minute crystals, sometimes arranged in thin platelets (Fujii, 1969). Other purines apart from guanine have been reported. .The presence of both guanine and hypoxanthine has been confirmed in the skin of coho salmon Onchorhynchus kisutch (Market and Vanstone, 1966) and in the Atlantic salmon (Johnston and Eales, 1967). The plate-like clefts, that have been constantly observed in the iridophores, seem to represent the spaces left by the guanine or hypoxanthine plates, possibly dissolved by tissue processing.

Hawkes (1974 b), in his account of the fish chromatophore unit in the coho salmon, described two types of iridophores. He claimed to have found a type of globular cell in the non-scaled head skin and in the layer above the stratum compactum. The fine structure of this cell was significantly different from the cells described as iridophores by other authors, as well as in the present study. It had a large electron lucent nucleus and a cluster of numerous mitochondria below the nucleus. The so called platelets were extremely narrow, arranged in stacks

which tangentially encircled the nucleus. The other type of iridophore described by Hawkes (1974 b) was not shown in the electron micrographs presented, but the author claimed it had an entirely different shape, with an elongated nucleus and mitochondria distributed throughout the cell. The platelets, however, were described as being about the same size as the others. This second type iridophore would have been found in the pigmented layer below the stratum compactum. It was not possible to confirm the existence of two types of iridophores in the course of the present work, because the observations concerning the dermis only included the stratum spongiosum above the scales.

The presence of inflammatory cells in the upper dermis in some fish was also reported in the present study. These inflammatory cells were lacking any sort of specific granules of the type described by various authors on fish granulocytes (Weinreb, 1963; Ferguson, 1976; Cannon et al. 1980). Their nuclear density and type of cytoplasmic inclusions, with distinct lysosome-like bodies suggest that they could be in fact macrophages. However, it is not the mere presence of these cells which seemed to be worth reporting, but the fact that they were present in large numbers.

To summarise the present discussion, it can be seen that a number of similar cell types was found both in the dermis and the epidermis, some of which were incidentally seen crossing the basement membrane. Epidermis and dermis, in spite of being quite distinct tissue types, displaying different functions, apparently also possess a degree of cellular exchange.

CHAPTER 3

DETECTION OF COMPLEX CARBOHYDRATES IN THE EPIDERMAL CELLS OF RAINBOW TROUT USING PHOSPHOTUNGSTIC ACID (PTA) AS A MARKER

Although in a nonspecific way, the phosphotungstic acid staining method, performed at a very low pH, can be a useful tool in the visualization of complex carbohydrates. The chemical significance of the PTA staining is still controversial, but the results obtained were analysed taking this into account.

3.1 - MATERIALS AND METHODS

Small fragments of skin (1 mm^3) were obtained from adult rainbow trout, and immediately immersed in the Karnovsky fixative, for 2 hours at 4°C. This fixation was followed by the usual washings in 0.1 M cacodylate buffer at pH 7.4. At this stage some of the material was submitted to a second fixation in 1% osmium tetroxide in the same cacodylate buffer, whereas other fragments were not post-fixed.

Dehydration in a graded ethanol series was followed by embedding in a mixture of Epon and Araldite, which was left to polymerize for 5 days at 45°C. The ultrathin sections were cut with an LKB Ultratome and collected in uncoated gold grids. The grids were subsequently floated, sections downwards, in the following solutions:

a) 1% periodic acid in double distilled water, for 30 minutes, or 10% hydrogen peroxide for 20 minutes, at room temperature. After this treatment the grids were thoroughly washed with water.

b) 0.5 N hydrochloric acid (HCl): 5 minute washing, at room temperature.

c) 4% PTA in 1N HCl, for 60 minutes at room temperature. The pH of the PTA solution was 0.25. The grids were carefully washed with water at the end of the treatment. No further staining was used, and the grids were examined in a JEOL 100 C electron microscope, at 80 Kv. The controls were not submitted to the 4% PTA solution, and were left floating on 0.5 HCl for 60 minutes, at room temperature.

3.2 - OBSERVATIONS

The results of the phosphotungstic acid (PTA) staining method varied with the ultrastructure of the skin fragments. Whenever the epidermis showed a normal appearance, with typical filament-containing cells (FCC's) and no intercellular spaces, their cell membranes showed occasional sites of dense staining (figure 70) and also small dense bodies, located throughout the cytoplasm (figure 71). No large dense bodies or phagosome-like vacuoles could be found in these normal FCC's. Whenever the epidermis showed extensive pathological effects, such as a major infiltration of mononucleated cells, the membranes of structures which seemed to be lysosomes, within presumptive macrophages, stained consistently with PTA (figure 72A). Such macrophages could easily be identified in the PTA treated sections, as well as those which were routinely stained (figure 72B).

The epidermal changes cited above were found not only in association with the macrophage infiltration, but also seemed due to a variation in the FCC's. In such cases, these cells showed a more rounded configuration, with large intercellular spaces and a decrease in the number of desmosomes. They also presented large vesicular structures which were seldom identified in normal cells (figure 53). The membranes of such vesicles were also stained with PTA, but not as densely as in the macrophages (figure 73).

The mucous vesicles of the goblet cells showed a variable response to the PTA staining. Within the same cell, some vesicles were densely stained, others only moderately stained and some did not stain at all (figure 74). No other internal structures of the mucous cells stained positively with the PTA, including the rough endoplasmic reticulum.

It was also possible to identify lymphocyte-like cells with dense nuclei in the epidermis . None of their internal structures showed positive PTA staining. The

clear cells with multilobulated nuclei already described in chapter 2 (figures 50, 51 and 52), showed the presence of PTA stained small dark granules and small vesicles throughout their cytoplasm (figure 75A). The dermal macrophages also had presumed lysosomes with PTA-positive stained membranes (figure 75B). On the other hand, the controls did not show any staining reaction, either in the mucous cells, or in the lysosomes. Even the desmosomes beween FCC's were totally unstained.

No particular difference was found in the staining intensity, whether the material had been pre-treated with periodic acid or with hydrogen peroxide. The tissue that had not been submitted to the osmium tetroxide post-fixation always showed more obvious staining.

Whenever the PTA staining was applied to skin sections of material which had been embedded using the routine procedure, i.e. post-fixed with osmium, embedded via propylene oxide and polymerized at 70°C, the results were negative, even for the mucous cells.

3.3 - DISCUSSION

Phosphotungstic acid (PTA) is no longer frequently used in electron microscopy staining of thin sections, having been successfully replaced by uranyl acetate and lead citrate (Pease, 1964). However PTA has deserved continuing interest because it can be sometimes used as a selective stain, demonstrating particularly extracellular tissue components and mucopolysaccharides of basement membranes (Pease, 1964). In fact, several attempts have been made to locate sites of periodic acid reactive carbohydrates in the electron microscope, following the work of Pease (1966) and Marinozzi (1968) who showed that phosphotungstic acid may stain carbohydrates in sections of tissues embedded in hydrophilic resines, at a very low pH. Since then, other experiments have followed these authors' work in animal tissues (Rambourg, Hernandez and Leblond, 1969; Babai and Bernhard, 1971; De Bruyn, Michelson and Becker, 1977; Barsotti and Marinozzi, 1980) and in plant cells (Roland, Lembi and Morré, 1972).

Espuelas, Navas, Bueno, Hidalgo and Lopez-Campos (1984) used several techniques to characterize the cells involved in carbohydrate production in carp (Cyprinus carpio) skin. One of the techniques was to stain ultrathin sections of resin-embedded tissue with 1% PTA in hydrochloric acid which, according to their account, produced similar results to the method of Thiery (1967).

In the present work,the skin fragments were embedded in Epon resin and the low pH was achieved with hydrochloric acid.

According to several authors' interpretations of the PTA method, complex carbohydrates were present in the areas where dense staining was obtained (Pease, 1966; Rambourg et al. 1969; Babai and Bernhard, 1971; De Bruyn et al. 1977). However, a certain controversy exists concerning the phosphotungstic acid stain, that certain authors claim not to be specific for polysaccharides (Glick and Scott, 1970;

Quintarelli, Cinfonelli and Zito,1971 a;Quintarelli,Zito and Cifonelli, 1971 b). Although not denying the results obtained by most authors who reported having stained polysaccharides, Glick and Scott (1970) maintained that the claim of a "polysaccharide stain" would only be substantial on the basis of a very large number of model experiments on pure substrates.

Contradicting these authors, Pease (1970) presented a series of arguments in support of the PTA as electron stain for complex carbohydrates. This author summarized most of the results obtained with PTA at low pH, concluding that it has become evident that the carbohydrate specificity of PTA was pH-dependent. He also added that the way through which PTA combines with exposed hydroxyl groups was still speculative. Alternating tungsten and oxygen atoms form a thick shell around a single phosphorus atom in the PTA molecule and the surface of the shell has 12 double bonded oxygen atoms, that might serve as potential hydrogen bonding sites, available to react simultaneously with the numerous hydroxyl radicals common to large carbohydrates (Pease, 1970). Furthermore, he did not preclude the hypothesis of PTA staining proteins at comparatively high pH, through ionic bonding of the phosphorus atom,which would not participate in the carbohydrate stain.

However, the basis for claiming that acidic, aqueous PTA is specific for complex carbohydrates is still empirical. In fact, studies on phosphotungstic acid stain by Quintarelli et al. (1971 a) using the polyacid at high concentrations and in extremely acidic conditions, succeeded in precipitating various compounds including proteins.

The precipitates obtained in this thesis were therefore interpreted as representing carbohydrates, although taking into account that some of the arguments presented against this idea seem to find reasonable support.

Carbohydrates seem to be associated with certain areas in the cell membrane of

the FCC's. However, the poor ultrastructural preservation makes it impossible to relate these membrane PTA-positive sites with desmosomes, although their distribution along the periphery of the cells seemed to suggest it. It was interesting to notice in those areas, the trilamellar pattern characteristic of the plasma membrane. Pease (1966) observed an interrupted layer of PTA-positive material between adjacent cells of the duodenal epithelium, but this material was clearly outside the cell membrane and not over it as in the present case.

De Bruyn et al. (1977) found that the surface of sinusoidal endothelial cells in rat were stained in a "spotty" fashion, only staining densely when in immediate contact with an underlying cell. However, the areas of high density found in the cell membrane by these authors showed an amorphous appearance, similary to what was interpreted as cell coat staining by Rambourg et al. (1969) in the cells of the kidney tubules, and did not display the trimellar structure found in the FCC's.

The small dense bodies and vesicles seen inside normal FCC's, sometimes assuming an elongated feature, suggested the existence of a scattered Golgi apparatus, which in fact was regularly present in these cells as small smooth surface vesicles and short saccules. Rambourg et al. (1969) found a gradient staining reaction of the Golgi stacks, the intensely staining side corresponding to the secreting mature side of the dyctiosome.

The PTA-positive sites in the FCC's membrane could possibly be associated with areas of exocytosis of carbohydrates, or carbohydrate associated substances, produced in the cells' Golgi apparatus and exported into the extracellular space. Espuelas et al. (1984) claimed that carp filament-containing cells produced sulphated acid glycoproteins, which were incorporated onto the cellular surface.

The vesicles with PTA-positive membrane identified in the present work in the cytoplasm of macrophages, cannot be unequivocally identified as lysosomes because

no histochemical identification of enzymes was undertaken. However ,their general size and their single unit membrane seems to suggest a lysosomal nature for these organelles.

As the lysosomes, in most cells, are formed and released from the mature side of the Golgi apparatus (Holtzman and Novikoff, 1984), their membranes have the same characteristics of the upper Golgi stacks. Therefore, it seems reasonable to expect them to display similar staining characteristics.

Rambourg et al. (1969) found numerous PTA-positive membrane vesicles as well as dense bodies in the rat duodenal epithelium, which they assumed to be lysosomes. The fact that the vesicles found inside FCC's, in special pathological situations, also showed PTA-positive staining in their membranes, may suggest a similar nature and origin of the above described vesicles in the macrophages. Their staining was however much less intense, similar to that found for secretory granules in the acinar cells of the pancreas by Rambourg et al. (1969).

Another possible interpretation could be that these vesicles were phagosomes, whose membranes are actually formed by endocytosis of the cell membrane (Holtzman and Novikoff, 1984). Activated macrophages frequently show phagosomes, or phagolysosomes when the cells are engaged in substantial phagocytic activity (Ghadially, 1982). The large vesicles with PTA-positive membrane within the FCC's could also be phagosomes, therefore representing signs of an eventual phagocytic activity of these cells. De Bruyn et al. (1977) described a variety of structures with PTA-positive membrane, in the rat endothelial cells, some of which were interpreted as organelles taking part in endocytosis such as coated vesicles and phagosomes.

It was not surprising to have found PTA-positive material in the mucous cells' secretory vesicles. Similar results were reported by Pease (1966) and Babaï and

Bernhard (1971) who studied the PTA staining on intestinal goblet cells. In both cases the staining was much more intense and Pease (1966) also noted a dense staining of the contents of the rough endoplasmic reticulum. However, this author used unfixed material and that might explain the differences observed. Babai and Bernhard (1971) also used unfixed frozen ultrathin sections. It is possible that some carbohydrates have been lost in tissue processing in the present work, although no osmium post-fixation was used.

The fact that Pease (1966) found presumed carbohydrates in the rough endoplasmic reticulum in the goblet cells is rather unexpected, considering the present knowledge of mucus synthesis in these cells. Assuming that glycosylation of the proteins produced in the reticulum is only achieved in the Golgi saccules (Berkaloff et al. 1978), no complex carbohydrates should be found in the exclusively protein producing areas.

The secretory vesicles of the carp mucous cells displayed an heterogenous reaction to some of the cytochemical techniques applied by Espuelas et al. (1984), similarl to the results of the PTA staining technique obtained in the present work.

Although controversial, the PTA staining produced some useful information concerning the nature of the cells found in the epidermis of rainbow trout, especially the filament-containing cells.

CHAPTER 4

BIOCHEMICAL PROCEDURES

4.1 - MATERIALS AND METHODS

4.1.1 - PREPARATION OF A RABBIT SERUM ANTI-TROUT IMMUNOGLOBULIN TO BE USED IN IMMUNOCYTOCHEMISTRY

NOTE - Several purification techniques are now available for the preparation of specific antibodies, the choice depending mainly on the availability of pure antigens in order to immunize the species selected for the antibody production.

The technique chosen in the present study used a simple process to obtain the purified antigen, linked to sheep red blood cells (SRBC). This choice was based on the difficulty of getting enough trout serum from which to purify the immunoglobulins, through the usual chromatographical or electrophoretical techniques. Rainbow trout were immunized with SRBC until a high antibody titre was reached. Their sera was then adsorbed onto SRBC, which, after suitable washing, were inoculated into rabbits. These produced an anti-IgM serum. The different steps used in the procedure were as follows:

4.1.1.1 - IMMUNIZATION OF TROUT WITH SRBC

Fish - Seven adult trout from TRUTURÃO (see chapter 1), two males and five females, weighing between 700 and 800 grammes, were used in the immunization procedure.

SRBC - Sheep's blood was collected from the jugular vein, defibrinated and kept at 4°C until use.

4.1.1.1.1 - IMMUNIZATION SCHEDULE

Five animals were injected intraperitoneally with 1 cc of a suspension of 5×10^8 SRBC in saline. Two controls were also injected in the same way with 1 cc of PBS, pH 7.4. Also for control purposes, two fish from the same group were bled prior to any immunization.

The blood was always collected from the dorsal aorta, at its median or caudal sections, with a sterile disposable syringe. The bleeding was always carried out under anesthesia with MS 222, and the blood was allowed to clot for one to two hours at room temperature and kept at 4°C overnight. The blood was then centrifuged at 2500 rpm for 20 minutes and the serum kept at - 20°C or -80°C before being used.

One month later the fish were reinjected using the same process. A week after this booster injection, two experimental and one control fish were bled. The four remaining animals were brought into the Veterinary School of Lisbon (chapter 1), where they were kept in a recirculating water system, using tap water, which was aerated for a period of 24 - 48 hours, in order to release any chlorine present, before being introduced into the system. During the experiment the fish were fed the same diet used at the farm. These fish underwent a third inoculation three weeks after the first booster, exactly as previously described. Two weeks later they were all bled.

4.1.1.1.2 - TITRATION OF THE TROUT SERUM ANTI-SRBC.

The complement of the sera was first inactivated at 44°C for 20 minutes (Sakai,1981). Using a microtest plate, 50 µl of saline were first introduced into each well. 50 µl of the sera under study were then added to the first left hand well of each row, and serially diluted with a semi-automatic device. Finally 50 µl of a suspension of SRBC, made by making a 2.5% dilution in saline of packed cells, were

added to each well.

The plates were left for an hour at room temperature.After this period, 50 µl of freshly obtained normal rainbow trout serum, diluted 1: 25 in saline, were added to each well, in order to provide the necessary complement for haemolysis to occur.

One hour later the results were read, being considered positive in all wells where haemolysis was shown. The reciprocal of the last dilution resulting in haemolysis was recorded as the titre. The sera showing the highest titres were used to adsorb onto SRBC, in order to inoculate rabbits.

4.1.1.2 - IMMUNIZATION OF RABBITS WITH TROUT IMMUNOGLOBIN ADSORBED ONTO SRBC.

The SRBC used for the first and subsequent injections in rabbits were prepared as follows:

a) The SRBC were washed three times in saline.

b) 0.5 cc of packed cells were gently mixed with 3 ml of undiluted rainbow trout serum and 10 ml of saline. The serum complement had previously been inactivated at 44°C for 20 minutes.

c) The mixture was kept at room temperature for two hours, with gentle frequent manual shaking.

d) The cells were subsequently centrifuged at 1500 rpm for 10 minutes, resuspended in 10 cc (total volume) of saline and again centrifuged, this washing process being performed three times. The final volume after resuspension in saline was 9 cc.

Three rabbits were inoculated with 3 cc subcutaneously at several sites along the dorsal surface. The use of adjuvant was considered unnecessary, as the whole SRBC were known to be quite immunogenic. In two of the rabbits 0.5 cc of the

inoculum was injected intramuscularly in the posterior limbs.

Three weeks later, a first booster was given, by injecting intramuscularly 0.5 cc of a SRBC suspension, prepared as previously described. A second booster was given three weeks later, and a third one three weeks after the second. The procedures were always the same as those described for the first booster. One of the rabbits died during the immunization process. Subsequently,the blood of the two remaining animals was used for serum collection. The rabbits were bled from the marginal vein of the ear in between inoculations and finally bled by cardiac puncture one week after the last booster. The blood was allowed to clot first at 37°C in an incubator, and subsequently overnight at 4°C. The serum was collected after centrifugation at 2500 rpm (20 minutes), and kept at -20 or -80°C, before being used.

The rabbit sera were tested for specificity by immunoelectrophoresis against trout whole serum as well as against purified trout immunoglobulin.

4.1.2 - PREPARATION OF RABBIT SERUM ANTI-WHOLE TROUT SERUM

NOTE - The preparation of this type of serum was considered important to test the degree of purity of the trout immunoglobulins prepared from whole trout serum using chromatographical methods.

Threerabbits were immunized using the following procedure:

- one rabbit was immunized intramuscularly with 2 cc of whole trout serum, mixed with 2 cc of Freund's complete adjuvant. Six weeks later, a booster injection was given with 1 cc of undiluted trout serum, administered intravenously;

- two rabbits were immunized intramuscularly with 4 cc each of an equal volume mixture of diluted trout serum (1:3) and Freund's complete adjuvant. A booster with 1 cc diluted trout serum (1:3) was given intravenously six weeks later.

The rabbits were bled a week after the booster, and the serum obtained was used in immunoelectrophoresis against whole trout serum. Two weeks after the first booster the rabbit immunized with whole trout serum was terminally bled by heart puncture and the two remaining rabbits were given a further booster with 1 cc of whole serum, administered intravenously. These rabbits were terminally bled by cardiac puncture one week later. As in all previous bleedings, the blood was allowed to clot at 37°C for one hour and overnight at 4°C. The following day, the serum was collected after centrifugation at 2500 rpm (20 minutes) and kept in the freezer at -20° or -80°C, until used.

The rabbits' response to the whole trout serum was tested by immunoelectrophoresis.

The agar (OXOID) was diluted in barbitone acetate buffer (pH 8.6). The trout serum migrated for 90 minutes at a suitable voltage. The diffusion was allowed to take place for 24 hours, and the slides, after another 24 hours wash, were dried in an incubator at 70°C and stained with coomassie blue.

4.1.3 - <u>PURIFICATION OF TROUT IMMUNOGLOBULIN (IgM) BY CHROMATOGRAPHY ON SEPHADEX G-200</u>

NOTE - The preparation of a small amount of purified trout immunoglobulin was considered important, to be used in testing the affinity and specificity of the rabbit sera.

Rainbow trout serum, obtained from animals previously immunized with SRBC, thought to have high immunoglobulin levels, was used in the purification process.

Prior to chromatography the serum immunoglobulins were precipitated with 40% ammonium sulphate. Once the procedure finished, the sample was dyalized

against water for 48 hours to remove sulphate ions and subsequently dyalized against the buffer used in gel filtration

Gel filtration on Sephadex G-200 (Pharmacia) was performed on a 2.5 x 100 cm column, in a cold atmosphere, with a 10mM phosphate buffer, pH 7.4.

The fractions of the eluted sample were automatically collected in an LKB -2070 ULTRORAC II. The elution profile was automatically registered with an LKB -2138 UVICORD S and an LKB -2065 CHOPPER-BAR RECORDER system. Some fractions of the eluted sample were considered individually while others were pooled, and immunoelectrophoresis against rabbit serum anti-whole trout serum was performed, in order to detect which fraction or fractions contained the purified trout immunoglobulins.

An immunoelectrophoresis of the eluted fractions considered to have trout IgM was run against rabbit serum anti-trout IgM (kindly provided by Dr. A.E. Ellis, Aberdeen), in order to confirm the presence of the immunoglobulins.

4.1.4. - ABSORPTION OF THE RABBIT SERUM ANTI-TROUT IMMUNOGLOBULIN

Defibrinated sheep red blood cells (SRBC) were thoroughly washed in saline and a 2.5% suspension was prepared with 0.15M PBS (pH 7.2). The SRBC were subsequently treated with tannic acid, in the same way as performed for passive haemagglutination tests (see 6.1.8).

The tannic acid SRBC were then coated with the proteins collected in the first fractions obtained from the Sephadex G-200 chromatography of the trout serum, presumably corresponding to the immunoglobulin fraction as indicated by the immunoelectrophoresis. The total protein of the fractions was determined by the colorimetric method for total proteins (Gornall, Bardawill and David, 1949), also

colorimetric method for total proteins (Gornall, Bardawill and David, 1949), also known as the biuret method, following the instructions of the laboratory which supplied the reagents (Laboratories Knickerbocker). The total protein determined was 0.55 mg/ml.

The coating was performed by adding to 2 cc of 0.15 M PBS (pH 6.4), 100 µl of the protein fraction and 0.5 cc of tanned SRBC. The mixture was left at room temperature for 30 minutes with frequent manual agitation. After this period the SRBC were centrifuged at 2500 rpm, washed with 1 cc of 0.15M PBS (pH 7.2) and centrifuged again. The supernatant was carefully removed, leaving the smallest possible amount of liquid. The coated SRBC were then resuspended in 0.5 cc of the rabbit serum anti-trout IgM, diluted 1 : 400, and left to incubate for 60 minutes at 37°C.

Following the incubation, the SRBC were removed by centrifugation at 2500 rpm and the serum carefully removed, in order to avoid the presence of any cells.

The serum prepared in this way was used in immunocytochemestry methods to control the specificity of the rabbit serum. It was used with the PAP method for light microscopy, replacing the primary antiserum (see 5.1.2).

4.2 - RESULTS

4.2.1 - PREPARATION OF RABBIT SERUM ANTI-TROUT IgM

4.2.1.1 -TITRATION OF TROUT SERUM ANTI-SRBC

The highest antibody titre obtained was 1024, from the serum of a fish which had been submitted to three intraperitoneal inoculations of SRBC. This serum was subsequently used to adsorb onto SRBC, in order to inoculate the rabbits.

The sera of control animals were negative.

The fresh serum proved to be a good source of complement and it did not produce haemolysis whenever added on its own to SRBC.

4.2.1.2-IMMUNOELECTROPHORESIS OF THE RABBIT SERUM ANTI-TROUT IgM

The presence of precipitating antibodies against trout IgM in the serum of the rabbits inoculated as explained in 4.1.1.2., was proved through the results of the immunoelectrophoresis against whole trout serum. A single precipitin line was developed at the site expected (figure 76), corresponding to the low mobility globulins.

4.2.2 -RABBIT ANTI-WHOLE TROUT SERUM

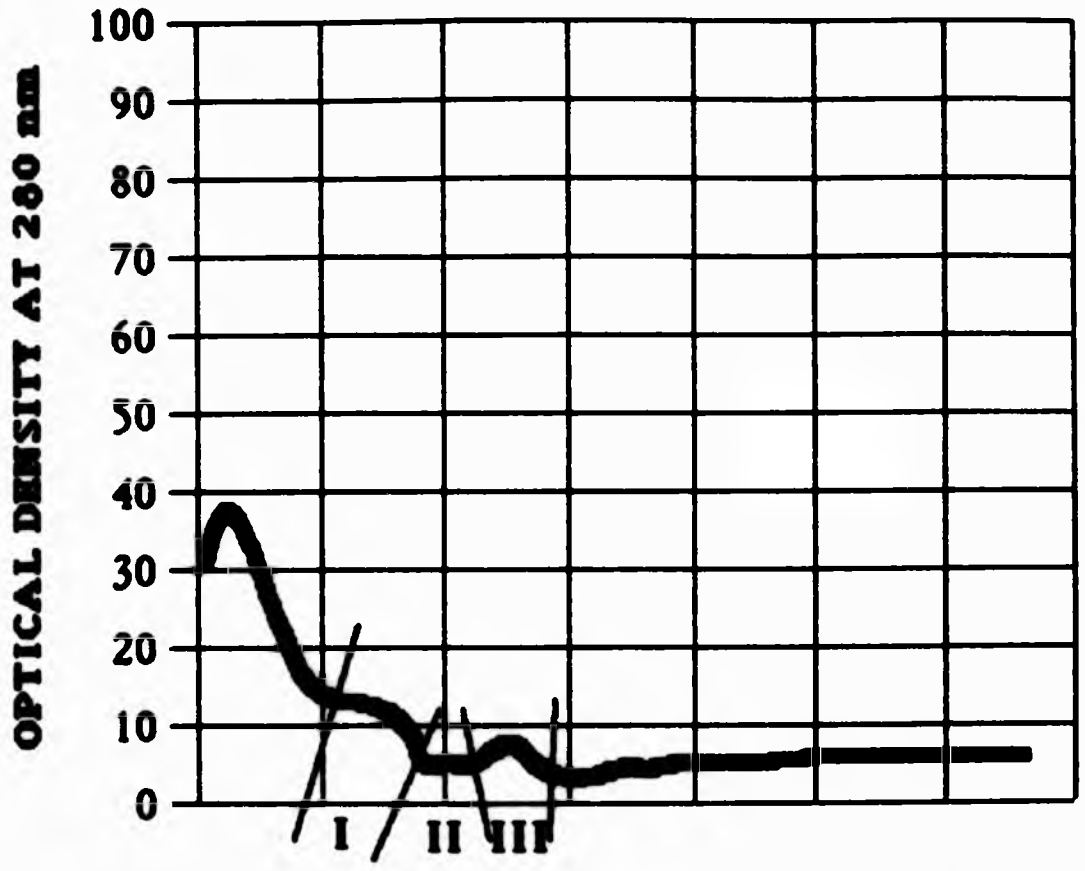
In the immunoelectrophoresis of the serum of the rabbits inoculated with whole trout serum, a series of precipitin lines was obtained (figure 77).

The serum of the rabbit inoculated with undiluted trout serum gave the best results.

4.2.3 - CHROMATOGRAPHY ON SEPHADEX G-200

The sample of ammonium sulphate treated trout serum resolved on Sephadex G-200 into three major peaks. The initial 18 fractions were individually analysed.

and three pools were made with the remaining eluates, numbered I, II and III, as indicated in the following profile.



Both fractions and pools were immunoelectrophoretically analysed with the rabbit serum anti-whole trout serum. Only the first four fractions gave a single precipitin line with the low mobility characteristic of the trout immunoglobulin (figure 78). They were subsequently analysed with the rabbit serum anti-trout IgM of Dr. A.E. Ellis, producing the same low mobility precipitin line. Therefore, they were considered as purified trout immunoglobulin.

4.3 - DISCUSSION

Sheep red blood cells have been widely used as antigens for rainbow trout (Chiller, Hodgins, Chambers and Weiser, 1969 a; Chiller, Hodgins and Weiser, 1969 b; Nonaka, Yamaguchi, Natsuume-Sakai and Takahashi, 1981 b; Blazer, Bennett and Wolke, 1984 ; St.Louis - Cormier et al. 1984), which seem to respond with high antibody titres (St.Louis - Cormier et al. 1984).

In their work on the complement system of rainbow trout, Nonaka et al.(1981b) found that haemolysis of sheep erythrocytes required both serum from trout immunized with SRBC and normal fresh rainbow trout serum. Incubation of sheep erythrocytes with heated rainbow trout serum alone, or normal rainbow trout serum alone, never induced haemolysis. This haemolytic activity in normal rainbow trout serum was identified as the fish complement system. The results of the present work totally agree with the findings of Nonaka et al.(1981 b). No haemolytic activity was found in the serum of the immunized fish prior to the addition of normal fresh rainbow trout serum, which was introduced as a source of complement.

The method chosen in the present work for obtaining rabbit serum anti-trout IgM was suggested some time ago by Milgrom, Luszczynski and Dubiski (1956) in order to prepare rabbit serum anti-human globulins. The same technique was followed by Ellis (1976) for the preparation of rabbit serum anti-plaice Ig . The only difference was that he used rabbit erythrocytes instead of sheep red blood cells.

The rainbow trout immunoglobulin was constantly referred to as IgM during the present work, because numerous structural studies concerning bony fish antibodies disclosed the existence of only one class of immunoglobulins similar to mammalian IgM and formed by four sub-units (for review see Corbel, 1975 and Dorson, 1981). Occasionally, however, some authors claimed to have found a low

molecular weight form of IgM, which would be present in the monomeric form in the serum of immunized fish (Clem and McLean, 1973; Lobb and Clem, 1981 a). Such a monomeric form was initially found in fish which had been immunized with haptens (Yocum, Cuchens and Clem, 1973).

The concept of two types of IgM was recently reinforced. Studies on anti-hapten immunoglobulins, isolated from sera of immunized rainbow trout, seem to have demonstrated the existence of two distinct IgM subpopulations, a monomeric and a tetrameric IgM, which differed in heavy chain peptide electrophoresis maps and in their immunoreactivity with rabbit serum anti-trout heavy chain, which was claimed not to be due to J-chain (Elcombe, Chang, Taves and Winkelhake, 1985). Furthermore, it was found that they also differed in their ability to activate complement, the tetrameric form being capable of activating both the classical and the alternative pathway, whereas the monomeric form would only activate the alternative pathway mechanism.

It may happen that in the near future the terms applied to designate fish immunoglobulins will be totally reorganized, especially following recent studies which have shown that catfish immunoglobulins are composed of heterogenous mixtures of covalent subpopulations (Lobb and Clem, 1983) and also that different light chain classes are present (Lobb, 1986).

The technique presently chosen for preparing the coated SRBC followed the directions of Williams and Chase (1967) for the isolation of specific antibody activity from intact erythrocytes. The only step avoided was the actual release of the antibodies from the erythrocyte membrane, because the blood cells were used as carriers of the antigen, with the increased advantage of avoiding the need for adjuvant, because the SRBC alone seem to be quite stimulating for the rabbit's immune system.

The precipitin line formed in the immunoelectrophoresis of the rabbit serum anti-trout IgM against whole trout serum developed at the site corresponding to the low mobility globulins and indicated by several authors as the site for trout immunoglobulins precipitation (Hodgins, Ridgway and Utter, 1965; Hodgins, Weiser and Ridgway, 1967; Dorson, 1972; Ingram and Alexander, 1979).

The precipitin pattern found in the immunoelectrophoresis of the rabbit serum anti-whole trout serum in the present work was identical to that which other authors have registered in similar circumstances (Dorson, 1972; Ingram and Alexander, 1979 ; Nonaka, Natsuume-Sakai and Takahashi, 1981 a).

The ammonium sulphate precipitation of immunoglobulins used in the present work to prepare the trout serum prior to the Sephadex G-200 chromatography was also carried out by St.Louis-Cormier et al. 1984, to purify rainbow trout serum immunoglobulin.

The elution profile of the trout serum from Sephadex G-200 obtained in the present work was similar to that which was found by other authors with immunized trout serum (Hodgins et al. 1967; Chiller et al. 1969 b; Ingram and Alexander, 1979). Antibody activity was found in the fractions of the first peak by Hodgins et al. (1967) and from the first and second peaks by Ingram and Alexander (1979). In the present study purified immunoglobulin was only found in the first fractions which corresponded to the ascending portions of the first peak, as was demonstrated by immunoelectrophoresis against rabbit serum anti-whole trout serum.

The method which was used in the present work to absorb the rabbit serum anti-trout IgM was based on the technique used to sensitize sheep red blood cells for passive haemagglutination tests. Antibodies or antibody fractions can be adsorbed onto sheep erythrocyte membranes, providing the cells are treated with tannic acid (Herbert, 1978). Although the amount of antigen added was very low (0.55 mg/ml).

this did not seem to be critical, for in many cases the range of antigen concentration suitable for sensitization was found to be quite large (Herbert, 1967). For coating sheep erythrocytes with antibody fragments the concentration of 1.0mg/ml was found satisfactory (Epstein and Gross, 1964).

As was reported in this chapter's results, this absorbed serum was used as control for specificity of the rabbit serum anti-trout IgM in the immuno-cytochemistry work. The absorbing technique used seems to have been successful as shown in the chapter 5 observations.

CHAPTER 5

IMMUNOCYTOCHEMICAL METHODS TO IDENTIFY IMMUNOGLOBULIN-CONTAINING CELLS IN THE EPIDERMIS

Immunocytochemistry seemed to be the most valuable technique available to detect the presence of immunoglobulin-containing cells, which would make possible the identification of such cells in ultrathin sections, observed with the electron microscope. This provided many problems in obtaining good labelling results at the ultrastructural level.

Postembedding staining was chosen and the method applied for light microscopy was the unlabelled antibody method of Sternberger (1974), also named the peroxidase anti-peroxidase (PAP) method. For electron microscopy the PAP was used as well as an immunomarking technique with goat serum anti-rabbit IgG, labelled with 10 nm gold probes.

5.1 - MATERIALS AND METHODS

Skin samples were obtained from the following fish:

Adults - Four specimens, one male and three females, from TRUTURÃO.

Juveniles - Eight juveniles, weighing between 60 and 300 grammes, from TRUTURÃO.

5.1.1 – FIXATION AND EMBEDDING PROCEDURES

From adults, 1 mm^3 fragments of dorsal, ventral and head skin were collected; fragments of the same size were obtained from the head and the lateral sides of the juveniles (figure 3). The samples were fixed in Karnovsky (2 hours at 4°C) or in 4% paraformaldehyde with 0.2% picric acid, in 0.1M cacodylate buffer, with 5 mM

calcium chloride ($CaCl_2$), pH 7.4. In this last method, fixation was carried out in an ice bath for 4 hours. The material fixed in Karnovsky was washed in 0.1 M cacodylate buffer, pH 7.4 after fixation, and part of it was post-fixed in 1% osmium tetroxide (OsO_4) in cacodylate buffer, for one hour at 4°C. Dehydration and embedding in a mixture of Epon and Araldite, followed the same schedule for the material with or without osmium post-fixation (room temperature):

70% Ethanol - 15 minutes;

95% Ethanol - 2 x 15 minutes;

100 % Ethanol - 3 x 15 minutes;

Mixture of 50% resin and 50% ethanol - 2x15 minutes;

Pure resin - 2 changes.

During the second change, the material was left overnight in a dessicator under vacuum. Embedding in rubber moulds or gelatin capsules was performed the following day. Polymerization was accomplished after 5 days in the oven at 45°C. The final consistency of this resin was markedly inferior to the one obtained with the routine procedure, using polymerization at 70°C, for 48 hours.

The skin fragments, fixed in 4% paraformaldehyde and 0.2% picric acid, were either embedded in Lowicryl K4M or in LR White resin (London Resin Company),according to the procedures described below.

After fixation the material was washed in 0.1 M cacodylate buffer, pH 7.4, with 5% sucrose (3 times 10 minutes and overnight). None of the material was fixed in osmium. For the Lowicryl, embedding, dehydration and infiltration were carried out in the following way:

30% Ethanol - 30 minutes at 0°C;

50% Ethanol - one hour at -20°C;

70% Ethanol - " " " ";

95% Ethanol - " " " ";

100% Ethanol -2 changes of one hour (-20°C);

Resin: Ethanol (1:1) - one hour at -20°C;

Resin: Ethanol (2:1) - " " " ";

Pure Resin - " " " ".

Polymerization was accomplished in gelatin capsules for 16 hours at -20°C, under ultraviolet light. For the preparation of the Lowicryl resin, the manufacturer's instructions were carefully followed.

The embedding in the LR White resin was followed up with three different procedures:

a) with accelerator, only ;

b) with accelerator and thermal cure at 50°C, overnight in the oven ;

c) without accelerator, in the incubator at 60°C, for 24 hours.

The dehydration was made complete in the same way for the three different procedures:

50% Ethanol - 15 minutes;

70% Ethanol - 2x30 minutes.

The infiltration of the material embedded with accelerator was performed with 3 one-hour changes in pure resin and final embedding in gelatin capsules. The resin was prepared according to the manufacturer's instructions, carefully mixing 10 ml of resin with a drop of accelerator, in a small beaker. Polymerization was noticeable around 10 minutes after the mixture was initiated. Therefore, no delays could be permitted whenever the accelerator was used. The whole procedure was carried out at room temperature. The material that was still to undergo thermal cure, at 50°C, was introduced in the incubator one hour after the polymerization was initiated with the accelerator.

The infiltration of the material that was only thermal cured followed a different process after the dehydration in 70% ethanol : two changes of pure resin, the first for one hour at room temperature, and the second, overnight, at 4°C. The fragments were finally embedded in gelatin capsules with pure resin, carefully filling them up to the brim and sliding the capsule cover on top, in order to limit contact with oxygen while polymerization occurred, as is advised by the

manufacturers. Such care is not necessary whenever the accelerator is used and consequently the resin is "cold" cured.

The semithin (2 μm) and the ultrathin sections of this material, were cut on an LKB Ultratome. The latter samples were collected on uncoated gold or nickel grids, as well as on formvar carbon coated nickel grids, accordingly to the material sectioned. The coated grids were only used to support skin sections embedded in Lowicryl or LR White. Such precautions were not found necessary whenever Epon-Araldite was the embedding medium.

Sections of Lowicryl embedded material were also collected in formvar coated copper 300 mesh grids. Most authors refer to the need of using nickel or gold grids for immunocytochemistry, but such a precaution is only necessary when oxidizing agents have to be applied to "soften the resin". When such a step is omitted, it is possible to use the less expensive copper grids.

The different types of sera used were obtained as follows:

a) rabbit anti-trout IgM, raised and tested as explained in chapter 4;

b) swine anti-rabbit immunoglobulins (DAKOPATTS, Denmark);

c) peroxidase-anti-peroxidase complex (PAP), raised in rabbit according to Sternberger,Hardy,Cuculus and Meyer,(1970) - (DAKOPATTS, Denmark);

d) goat anti-rabbit IgG, labelled with 10 nm colloidal gold (EMGAR-10) - JANSSEN LIFE SCIENCE PRODUCTS (Belgium) ;

e) normal rabbit, swine and goat sera were obtained by bleeding healthy and non-inoculated animals from the cited species.

All sera were kept in a freezer at -80°C, after dilution, except for the EMGAR-10, which was kept at 4°C.

5.1.2 - LIGHT MICROSCOPY IMMUNOSTAINING WITH THE PAP METHOD

NOTE: There is a great advantage in staining thick sections for light microscopy observations, before moving forward to ultrastructural observations. The presence of antigen in the tissue under study can be easily tested, as well as its resistance to the whole processing scheme. The antiserum affinity can also be evaluated, and the optimal dilution determined, according to the light microscopy staining results.

The peroxidase anti-peroxidase (PAP) or unlabelled antibody method of Sternberger (1974) was chosen for these studies.

Two micron sections of Epon-Araldite resin embedded material were mounted on glass slides, which were especially prepared for good adherence by immersion for a few minutes in the following freshly prepared solution:

1 g of gelatin;
0,1 g of chrome alum;
200 ml of distilled water;

After immersion the slides were left to dry, without touching each other, for 24 hours.

The sections were collected in distilled water and left to dry on the hot plate for 30 minutes, at 40 - 45°C. Afterwards, they were left in the incubator, at 37°C, for two days, before proceeding with the staining.

The resin was always removed with a saturated solution of sodium hydroxide in absolute ethanol (prepared at least a week before use, and no more than two months old). After resin removal, the sections were hydrated and endogenous peroxidase

was blocked with hydrogen peroxide (H_2O_2). The blocking of unspecific proteins was also carried out with 10% normal swine serum, in 50 mM Tris(HCl)buffer, pH 7.6 in 0.15 M sodium chloride (tris saline). This buffer was the same as that used for the washings and to dilute the rabbit serum anti-trout IgM (RS-TIgM) in a series of 1: 10; 1: 50; 1: 100, 1: 200; 1: 400; 1: 800; 1: 1600 and 1: 3200. The incubation took 20 hours at 4°C, in a moist atmosphere, with another two hours at room temperature (r.t.), to allow equilibration.

A swine serum anti-rabbit IgG was subsequently used, in a 1: 30 dilution in the usual tris saline buffer, for 45 minutes (r.t.).

Incubation with the peroxidase-antiperoxidase complex, diluted 1: 80 in tris saline, took one hour (r.t.). The slides were washed in buffer, and the peroxidase activity was demonstrated by the Graham and Karnovsky method (1966; cf. Sternberger, 1974 and Larsson, 1981).A solution of 100 cc of 0.05% 3,3' – diamino-benzidine (3,3', 4, 4' tetramino biphenyl) tetrahydrochloride (DAB, SIGMA), in tris saline with 0.01% H_2O_2 was prepared and the slides left to soak for 30 minutes. They were then thoroughly washed in distilled water and stained with Erlich's haema-toxylin for 10 minutes. Dehydration and mounting were completed as in any routine staining procedure (see appendix for details on the methods).

Controls included staining with normal rabbit serum,used at several dilutions, replacing the RS-TIgM, as well as omitting the primary antibody step. Staining with absorbed RS-TIgM (see 4.1.4) was also carried out to control its specificity.

The presence of endogenous peroxidase in the tissue sections was determined by incubating them, after resin removal, in 0.05% diaminobenzidine with 0.01% H_2O_2.

5.1.3 - ELECTRON MICROSCOPY IMMUNOSTAINING

The skin fragments, embedded in the Epon-Araldite resin mixture, were stained with the PAP method as well as with the immunogold labelling method. The material embedded in Lowicryl and in LR White was submitted only to the latter staining procedure.

5.1.3.1 - PAP IMMUNOLABELLING METHOD

The nickel or gold grids were always etched by floating them, sections down, on small droplets of 10% H_2O_2 for 5 minutes. Osmium fixed material was also etched, but for 20 minutes.

After this, the grids were washed with double distilled water (delivered as a stream over the grid, using a pipette) and with 50 mM Tris(HCl) buffer, pH 7.6, in 0.15 M sodium chloride (tris saline), the same buffer used for light microscopy staining. From this step onwards the grids were never allowed to dry completely, although they were blotted, touching their edge against filter paper. After etching they were exposed to an inert protein solution. In this case, as in the previous method described, normal swine serum was used, diluted 1: 10 in tris saline, for 30 minutes (r.t.). Grids were again blotted against filter paper and floated on droplets of the solution of the RS-TIgM. The serum dilution used was 1: 400 in tris saline and incubation took 20 hours at 4°C plus 2 hours at room temperature.

After rinsing in buffer the sections were again exposed to the 1: 10 dilution of normal swine serum for 30 minutes, blotted and floated on the swine serum anti-rabbit IgG , at a 1: 30 dilution, for 30 minutes. This was followed by a new rinse in tris saline and a new incubation in the normal 1: 10 swine serum for 30 minutes, before exposing them to the PAP 1: 30 dilution. The PAP complex was diluted in tris saline with 1% normal swine serum and the grids were kept floating for 20 minutes.

after which they were thoroughly rinsed in the buffer.

Peroxidase activity was demonstrated by incubating the grids in a freshly prepared solution of 0.0125% diaminobenzidine tetrahydrochloride (DAB), with 0.0025% H_2O_2, in the same tris saline buffer used throughout the method (Sternberger, 1974). A more concentrated solution of 0.05% DAB, with 0.01% H_2O_2 in tris saline was also used, for the same demonstrating purposes.

Incubation time was 3 minutes, either by floating the grids on small containers with the DAB solution, or by dipping them in a beaker containing the solution, kept under constant agitation over a magnetic stirrer. This last method almost invariably led to the formation of large amounts of precipitate, which adhered to the grids. Once the three minute incubation was over, the grids were immediately immersed in double distilled water, thoroughly washed and finally dried.

The diaminobenzidine polymers were then fixed with osmium tetroxide, by floating the grids on a 4% solution in double distilled water, for 20 minutes (r.t.).

Staining with uranyl acetate or lead citrate was not used in order to allow a better identification of the electron dense polymers.

5.1.3.2 - IMMUNOGOLD LABELLING METHOD

The procedure used is relatively simple, for the gold particles are easily visible in the electron microscope and there is thus no need for a developing process like that used in the PAP method.

The sections embedded in the Epon-Araldite resin mixture were etched, either with 10% H_2O_2 (5 minutes for non osmicated specimens) or with a saturated aqueous

solution of sodium metaperiodate (MERK), for 60 minutes.

Another step was introduced for some of the grids: incubation in a solution of 0.005 mg/ml of a nonspecific protease (type VII from Bacillus amiloliquefaciens, SIGMA) in PBS, PH 7.4, for 3 hours at 37 °C.

So, the following combinations of etching and protease treatment were tried:

a) for osmicated specimens:

1 - sodium metaperiodate and protease treatment;

2 - sodium metaperiodate only .

b) for non-osmicated specimens:

1 - sodium metaperiodate and protease treatment;

2 - sodium metaperiodate only;

3 - 10% H_2O_2 and protease treatment.

The grids were always thoroughly washed after etching with double distilled water. If the protease treatment had been applied the grids were washed with PBS.

From this moment onwards the technique was the same for all specimens, no matter what type of resin had been used. The Lowicryl or LR White grids did not need to be "deplasticized".

The RS-TIgM was diluted 1: 400 in a 0.1% BSA -tris buffer (20mM tris buffered saline, supplemented with 1mg/ml BSA, pH 8.3), with 1% normal goat serum. The same buffer, without the normal goat serum, was also used to dilute the other sera as well as for washing purposes. The RS-TIgM was applied for two hours (r.t.), after a short incubation (15 minutes) in 5% normal goat serum, to inhibit nonspecific protein reactions. The gold labelled goat serum was used at a 1: 25 dilution, for one hour (r.t.).

The grids were contrasted with uranyl acetate and lead citrate as is usual for electron microscopical observations.

The controls included the use of normal rabbit serum, diluted 1: 400 in 0.1% BSA-tris saline buffer, to replace the RS-TIgM . The nonspecific affinity of the gold labelled goat serum towards the skin sections was also tested by omitting the incubation with RS-TIgM.

All observations were made in a JEOL 100 C electron microscope at 80 Kv.

5.2 - OBSERVATIONS

5.2.1 - LIGHT MICROSCOPY OBSERVATIONS

Numerous immunoglobulin-positive cells were detected in the skin sections through the presence of dark brown deposits (figures 79, 80 and 82), even at high anti-serum dilutions. Most positive cells could not be clearly identified. The exceptions were the mucous cells, in which the degree of staining was not always the same. They varied from unstained to totally or partially heavily stained. The immunodeposits seemed to be located all over the positive cells' cytoplasm and not restricted to the cell membranes. The malphigian cells were generally negative, although, in some cases, they displayed small dark deposits (figure 79). Intercellular immunodeposits were also occasionally located (figure 82), being more obvious in the lower epidermis. In the dermis, small positive cells were also identified between the collagen bundles (figure 82).

The controls were consistently negative, either when the staining was performed with the RS-TIgM previously absorbed with the antigen (figure 83) or when the RS-TIgM was replaced with normal rabbit serum (figure 81). The sections treated with diaminobenzidine to detect the degree of endogenous peroxidase did not show any brown deposits.

5.2.2 - ELECTRON MICROSCOPY OBSERVATIONS

5.2.2.1 - SECTIONS TREATED WITH THE PAP METHOD

In spite of the good ultrastructural preservation of the material treated with the PAP method, results were always difficult to interpret. Although the sections were unstained, the epidermal cells were easily identified. Interpretation problems were mainly due to the precipitate that regularly covered the sections. The reaction

product is known to show up as black deposits, so it became particularly difficult to differentiate what was positive material or just nonspecific and therefore unwanted precipitate. Such precipitate was formed no matter which concentration of DAB had been used (0.025% or 0.05%). In one case, however, it was possible to locate unequivocally positive mucous cells, as well as small dark granules in the cytoplasm of the FCC's. Such dark granules did not seem specifically related to any cell organelle (figure 84).

5.2.2.2 - SECTIONS TREATED WITH THE GOLD-LABELLED ANTIBODY METHOD

The material fixed with Karnovsky and embedded in Epon-Araldite showed a very good preservation of the ultrastructure, as was previously mentioned in 5.2.2.1. However, it was not possible to identify any sites of labelling, for only few gold particles were seen regularly dispersed throughout the sections. Therefore, the distribution observed for the label was considered as nonspecific. No particular difference was noticed in the material submitted to etching procedures, such as H_2O_2 or sodium metaperiodate. The protease treatment similarly did not result in increased labelling.

The observation of the sections used as control of specificity showed similar results to the experimental ones, i.e. few gold particles, ascertaining the nonspecificity of the labelling.

The material fixed in Karnovsky and embedded in Lowicryl showed a poor, but still acceptable, degree of preservation, for the cells could be easily identified, including some intracellular ultrastructural details. The labelling obtained corresponded to what seemed to be lymphocyte-like cells (figures 85 and 86) and mucous cells. In the first case, the label was dispersed through the cytoplasm, and the poor preservation did not allow the possibility of localizing the gold particles over any of the cell organelles. However, it was evident that the cell membranes

were not particularly labelled. Over the nucleus and the extracellular space, a negligible number of gold particles were present. Some apparently multinucleated cells also showed gold particles within their cytoplasm, similar to that observed for the lymphocyte-like cells (figures 87 and 88).

The mucous cells, mature or immature, were intensely labelled, with the particles condensed within the mucous vesicles (figures 89 and 90). Neither the rough endoplasmic reticulum of these cells nor other cell structure were labelled.

Labelled cells were also found in the dermis of the experimental skin sections, with particles dispersed through the cytoplasm. The ultrastructure of these cells seemed to correspond to macrophages, with a moderately dense nucleus and lysosomal-like bodies in their cytoplasm (figure 91).

The controls demonstrated the specificity of the method as no labelling was obtained in the sections where normal rabbit serum was used in replacement of the RS-TIgM (figure 92), nor in the sections where this first step was omitted.

The material fixed in paraformaldehyde and picric acid, and embedded in Lowicryl, showed an inferior preservation of the ultrastructure compared to the previous fixation method. However, the antigenicity seemed reasonably preserved, for some lymphocyte-like cells were clearly labelled (figures 93 and 94). In these cells the particles seemed regularly dispersed, with no specific location in the cytoplasm, mainly due to the poor preservation of the material. The cell membranes did not show any particular labelling. A few particles were seen over the nucleus, but their scarcity was interpreted as nonspecific labelling. The mucous cells were not as heavily labelled as in the Karnovsky fixed material. The gold particles seemed to be located in between the mucous vesicles and not inside them.It should be noticed that the skin samples did not always belong to the same specimen, therefore some individual variability was to be expected.

The controls proved the specificity of the reaction, showing no labelling, as confirmed in the previous case.

The material fixed in Karnovsky and embedded in LR White constantly showed very poor preservation of the skin ultrastructure. Most of the labelled cells had their cytoplasm disrupted, making their identification very difficult. In spite of the three polymerization processes used, the results were equally poor regardless of the method.

The skin fragments fixed in paraformaldehyde-picric acid mixture and embedded in LR White, showed the lowest quality of ultrastructural preservation of the different methods used in order to prepare the material for immuno-cytochemistry.

Gold particles were present in the cytoplasm of some cells but the labelling was not very marked (figure 95). In spite of the difficulty in recognizing the internal structure, the size of the labelled cells and their nucleus suggested that they were lymphocyte-like cells. The mucous cells of the same material whose description was previously made for the Lowicryl embedding, did not show the same intense labelling (figure 96). As in the previous case, no differences were noticed between the labelling in sections of material submitted to the various polymerization methods used.

The controls contributed, as previously, to prove the specificity of the method, showing no labelling in the sections used for this purpose.

5.3 - DISCUSSION

5.3.1 - LIGHT MICROSCOPY

The presence of immunoglobulin (Ig)-positive cells in the epidermis of rainbow trout was consistent with the results previously obtained by the present author in paraffin-embedded skin fragments (Peleteiro and Richards, 1985). The fact that the reaction remained positive in the resin-embedded skin sections proves that the fish Igs retained their antigenicity through the fixation and embedding procedures used in this work.

Although the type of positive cells was generally not identifiable with certainty, their size and location related them to the small cells with dark staining nuclei usually observed in the epidermal sections in the light microscope. Some positive cells in the lower epidermis, especially the larger ones, could also be immature mucous cells. The presence of Igs in the mucous content of the goblet cells is in agreement with the findings of some authors who detected antibodies in the mucus of rainbow trout (O'Rourke, 1961; Fletcher and Grant, 1969; Di Conza,1970; Bradshaw et al. 1971; Di Conza and Halliday, 1971; Fletcher and White, 1973; Harrel et al. 1976; Ourth, 1980; Lobb and Clem, 1981 b; St.Louis-Cormier et al. 1984). The differences in labelling of the mucous cells found in the present work may indicate some variability of the antibody concentration in the mucus.

The immunocytochemical staining was followed up in ultrathin skin sections to allow a more clear identification of the positive cells, as well as of the location of the Igs within the cell organelles.

5.3.2. - ELECTRON MICROSCOPY

The best results of ultrastructural localization of rainbow trout Ig in the

epidermis were obtained with material fixed in Karnovsky, embedded in Lowicryl and stained by the immunogold labelling method. The Karnovsky fixative preserved the Ig antigenicity, as was already shown by the light microscopy results. On the contrary the use of the paraformaldehyde - picric acid mixture produced very poor ultrastructural preservation and did not result in increased antigenic response, in spite of previous favorable reports on postembedding staining of mammalian Igs (Takamiya, Batsford and Vogt, 1980).

Other fixatives, however, should be tested for future application of immunocytochemical methods in order to obtain the best compromise between ultrastructure and preservation of antigens. Such problems do not have to be faced in preembedding staining, which, in turn, can only be successful if a good penetration of antibodies or antibody markers is achieved in the thick sections usually employed. Preembedding seems to be more suitable for surface labelling of isolated cells. It is presently known that antigens can be resistant to osmium fixation as some postembedding staining experiments have proven (Bendayan and Zollinger, 1983).

The Lowicryl as embedding medium showed to be superior to the Epon in the present immunogold labelling experiments. Two factors may have influenced the final results: a higher penetrability of the hydrophilic resin to the antibodies and a better preservation of antigenicity. Roth, Bendayan, Carlemalm, Villiger and Garavito (1981) compared the suitability of Lowicryl and Epon for postembedding staining of thin sections, with the protein A-gold technique. These authors found an enhancement of the general cellular fine structure and an improvement of the immunocytochemical labelling in the material embedded in Lowicryl. Such improvement was attributed to the low temperature of the embedding procedure.

The results obtained in the present work through the use of LR White resin as embedding medium were not very encouraging. However, it is possible that the

resin was polymerized at a too high temperature. Newman, Jasani and Williams (1983) reported good results in immunostaining procedures using LR White, which was polymerized at 50°C for 24 hours, under anaerobic conditions. In fact, the accelerator that was used in the present work produced a highly exothermic polymerization that was concluded in 20 minutes. It was not possible to control the temperature achieved in the procedure and it may well have surpassed 60°C.

The LR White resin presents several advantages to its use that should be considered, such as: it tolerates partial dehydration and no etching procedures are necessary. Also the penetrability of the resin seems to be equivalent to the other hydrophilic resins. Therefore, further attempts should be made to test its suitability in spite of the unfavourable results presently obtained.

The immunostaining of the Epon-Araldite embedded material produced the worst results, especially with the immunogold labelling method. It is believed that the main reason was the poor penetrability of the antibodies together with a certain degree of nonspecific staining.

This problem may be related to the strong absorption of many proteins which seems to occur with ultrathin sections of Epon and methacrylate as reported by Sternberger (1974) or Epon 812 and Araldite as reported by Larsson (1981). To overcome this disadvantage the thin sections were pretreated with normal serum of the species delivering the second antibody, as recommended by these authors, but nonspecific staining was not abolished.

Several authors reported good immunocytochemical results using different postembedding staining methods in material embedded in Epon (Nakane, 1971; Roth, Bendayan and Orci, 1978; Takamiya et al. 1980; Nicosia, Sowinski, Chilton and Streibel, 1984) or Araldite (Silverman and Zimmerman, 1975). However, none seems to have used Epon-Araldite mixtures, which could in fact be more difficult to

penetrate and more susceptible to induce nonspecific staining. Takamiya et al.(1980) reported the successful use of nonspecific protease to restore the protein antigenicity of plastic embedded material. According to some authors, the most important reason for the lack of good immunocytochemical staining was the chemical modification of the antigenic determinants by plastic monomeres (Vogt, Takamiya and Kim, 1976). However, in the present case, the use of a type VII protease from Bacillus amiloliquefaciens did not improve the final results. A strong oxidizing agent such as sodium metaperiodate was used by Bendayan and Zollinger (1983) for ultrastuctural localization of antigens in osmium fixed tissues. This same oxidizing agent was used in the present work in Epon-Araldite embedded material, but the final results were as unsatisfactory as the ones obtained by the use of 10% H_2O_2 as etching agent.

The presence of Igs in the lymphocyte-like cells found in the epidermis was not surprising. Many authors have reported the presence of Igs in the cell membrane of lymphocyte-like cells in fish (Ellis and Parkhouse, 1973; Emmrich, Richter and Ambrosius, 1973 ; Ellis, 1976; Warr, De Luca and Marchalonis, 1976; Clem, McLean, Shankey and Cuchens, 1977 ; De Luca, Warr and Marchalonis, 1978), namely rainbow trout (Etlinger, Hodgins and Chiller, 1977 ; Warr, De Luca and Griffin, 1979).

All above mentioned authors used cell suspensions and immunofluorescence labelling methods with polyclonal rabbit serum. The results presented were very homogenous, with virtually all lymphocytes showing membrane Igs, with no significant differences in labelling having been found between spleen, kidney, thymus or circulating lymphoid cells. An exception should be made for Emmrich et al. (1973) who found a very high percentage of positive cells in the carp thymus, followed by the pronephros and blood lymphocytes and finally the spleen with the lowest number of cells bearing surface Igs. De Luca et al. (1978) reported in Carassius auratus and C. carassius a different hierarchy of fluorescence intensity.

being higher in the spleen lymphocytes, followed by the head kidney and finally the thymus.

The surface labelling of thymocytes obtained by most authors with polyclonal rabbit serum anti-trout Ig was meanwhile questioned by Yamaga, Kubo and Etlinger (1978) who detected cross reactivity between trout Ig and keyhole limpet hemocyanin (KLH). Following these findings,they claimed that no conventional Ig was found on thymocytes and that most reactivity was due to carbohydrate moieties.

In most of the research work that subsequently followed with monoclonal antibodies, different results were obtained in what concerned surface labelling of lymphocytes. Lobb and Clem (1982) and Sizemore, Miller, Cuchens, Lobb and Clem (1984), using catfish (Ictalurus punctatus) peripheral blood and splenic lymphocytes, showed that monoclonal antibodies against catfish Igs only detected 40% positive cells with membrane Ig. In rainbow trout similar results were achieved by De Luca, Wilson and Warr (1983) using monoclonal antibodies to trout IgM. They found that the percentage of cells with membrane Ig was quite variable, very low in the thymus (around 5.2%), higher in the head kidney (around 12.4%) and reaching the maximum in the spleen (around 30.3%).

Such a clear separation of Ig membrane-positive and negative cells was not found in the carp by Secombes, Van Groningen and Egberts (1983) who also worked with monoclonal antibodies raised against carp thymocytes and carp serum Ig. They produced some antibodies that were positive for thymocytes and for serum Ig and which stained most cells in the thymus. Ambrosius, Fiebig and Scherbaum (1982), working on carp thymocyte receptors, proved the existence of a thymocyte membrane Ig, which would be composed of two heavy chains, linked together by disulphide bridges and non-covalent bonds. No light chain components were found to take part in this membrane Ig determinant, which could explain why only monoclonal antibodies reacting against the variable region on the Ig heavy chain

were binding to carp thymocytes as was found by Secombes et al. (1983). For Ambrosius et al. (1982) these membrane determinants in thymocytes would very likely be antigen-specific receptors as was shown by functional studies. They also proved that, for carp, carbohydrates did not play an important role in the antigenic cross-reactivity between IgM and the thymocyte membrane determinants. Unfortunately, no special determinants have yet been shown for trout thymocytes.

The identification of subpopulations of fish lymphocytes based upon their response to mitogens has been shown by various authors in rainbow trout (Etlinger, Hodgins and Chiller, 1976; Warr and Simon, 1983), in the bluegill Lepomis macrochirus (Cuchens and Clem, 1977) and in the channel catfish (Sizemore et al. 1984). Although the results were not always the same in what regards the organ distribution of the mitogenic response, no doubts seem to have remained about the real existence of two subpopulations of lymphocytes identical to what was described for the rainbow trout by the above cited authors. De Luca et al. (1983). They reported a clear response to lypopolysaccharide (LPS) by the head kidney lymphocytes bearing surface Ig and a response to concavalin A (ConA) by the lymphocytes with no surface Ig.

In many aspects it seems possible to establish a close correlation between lymphocyte subpopulations in mammals and in fish, with a high predominance in the latter of T-like cells in the thymus, B-like cells in the anterior kidney and a mixed population in the spleen and blood. However, other important data are missing, such as identification of C3 or Fc receptors, which have not yet been found in fish lymphocytes or macrophages (Wrathmell and Parish, 1980).

Although, for mammals, the presence of intracytoplasmic Ig is thought to be characteristic of the B lymphocytes, it should not be assumed that the same is valid for fish. Intracytoplasmic Igs were identified in carp peripheral blood lymphocytes by Caspi, Shahrabani and Avtalion (1980), through immunofluorescence methods.

These authors found a significant difference between the results obtained with rabbit serum anti-carp Ig absorbed with homologous erythrocytes and with the same serum absorbed with autologous red blood cells. They subsequently suggested the existence of differences in Ig determinants among carp peripheral blood lymphocytes.

The functional separation of T and B-like cells has not yet been fully established in this group of lower vertebrates, altough recent studies by Miller, Sizemore and Clem (1985) on anti-hapten responses to thymus-dependent and thymus-independent antigens of catfish lymphocytes provided strong evidence that these fish have separable lymphocyte populations, B cells (positive for surface Ig) and T helper cells (negative for surface Ig).

The identification of Igs in the cytoplasm of lymphocyte-like cells in the epidermis of rainbow trout does not seem enough to include these cells in any particular lymphocyte subpopulation. The fact that no significant labelling was found in the membranes of these cells could lead to their identification as surface Ig negative cells. However, the loss of cell surface antigens during fixation and embedding of the tissue might have occurred, explaining the low degree of labelling achieved.

It should also be noted that the immunocytochemical experiments carried out in the present thesis have not been applied to cell suspensions. Therefore, only superficial antigens in the sections were available for labelling, this including antigens in the cytoplasm. These are usually not accessible whenever cell suspensions are stained. This might explain why no cytoplasmic determinants were ever detected in fish lymphocytes, because most authors used cell suspensions of the various organs and blood cells for immunolabelling. The ultrastructural immunocytochemical studies carried out by Booyse, Sternberger, Zschocke and Rafelson (1971) on human platelets produced different results whether intact cells or

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cell sections were being used. Antibodies were unable to penetrate intact fixed platelets, even after prolonged incubation.

Ig-positive macrophage-like cells were also noticed in the skin sections, particularly in the dermis, in the course of the present work. Earlier studies, in rat, on antibody producing cells, in which labelling methods were used to trace intracellular Igs, have shown that monocytes and macrophages can also present a certain degree of labelling (Bosman, Feldman and Pick, 1969; Bosman and Feldman, 1970). The Igs found within the macrophages were thought not to be associated with antibody synthesis (Bosman et al. 1969).

The same is probably true for fish, although some reports exist on the close involvement of macrophages in the behaviour of lymphocytes of channel catfish by Sizemore et al. 1984. They found that the lymphocyte subpopulation without membrane Ig did not respond to LPS or ConA unless macrophages were present, in which case response was obtained to both mitogens. It was concluded that macrophages acted upon lymphocytes as accessory cells. The Igs in the macrophages could have been endocytozed from the extracellular space where it seems to exist at a certain concentration. Ig-positive cells in the dermis had previously been reported in the light microscopy results.

More controversial is the degree of labelling that was found in some epidermal mucous cells. In the light microscopy results a variable degree of staining of these cells was reported. In the ultrathin sections the degree of labelling was also variable, even in material submitted to the same fixation and embedding procedures. This suggests that the labelling was not due to cross-reaction with mucus glycoproteins in the secretory vesicles, although this possibility cannot be completely ruled out. One of the sugars identified in the Atlantic salmon mucus was fucose (Harris and Hunt, 1973). L-fucose was found to be the carbohydrate associated with rainbow trout immunoglobulins which would cross-react with KLH

(Yamaga et al. 1978). However, fucose was certainly also present in most mucous cells content, and only a restricted number of them was heavily labelled.

If the labelling was in fact demonstrating the presence of Igs in the mucous cells the question arises about how and where were they produced? In fact, the only cells in the epidermis which resemble plasma cells are the immature mucous cells.It is the high development of the rough endoplasmic reticulum (RER) that is common to both cell types. Fish plasma cells are very similar to the mammalian counterpart (Smith et al. 1970; Peleteiro, 1981). However, no labelling was found in the RER of the positive mucous cells, suggesting that the antibody was not being produced in situ.

It could also happen that the antibody was in fact being produced in the RER of the mucous cells, their carbohydrate associated molecules being added at the final stage of formation of the secretory vesicles. This would explain the lack of labelling in the RER, but it should meanwhile be assumed that no cross-reaction was taking place with other carbohydrate molecules.

Another explanation for the presence of Igs in the mucous cells could be the absorption by these cells of the antibody present in the extracellular epithelial space, through endocytosis, to become incorporated in the mucous secretion.

An interesting concept about intestinal production of secretory IgA was advanced by Clamp (1981). Whenever comparing the monosaccharide units of IgA, before and after entering mucous secretions, it was noticed that additional monosaccharide residues had been added, forming what was called a "mucus-like" stretch in the hinge region of the Ig. It was consequently suggested that one possibility could be that the IgA molecule was additionally processed during passage through some second cell.

It could be argued that the fish tetrameric Ig is too large a molecule to be

absorbed by any particular cell. However, evidence is growing on the existence of other types of Ig, namely a monomeric one (Lobb and Clem, 1981 a; Elcombe et al. 1985) which could easily be endocytosed.

Immune complexes seem to act upon the intestinal mucosa producing mucus release from goblet cells (Walker, Wu and Bloch, 1977). If this were also true for fish skin, the presence of antibody in the mucous cells could increase the efficacy of the mucus in destroying and removing pathogens.

CHAPTER 6

DETECTION OF ANTI-VIBRIO ANGUILLARUM ANTIBODIES IN THE EPIDERMIS OF RAINBOW TROUT VACCINATED BY DIRECT IMMERSION

Although the presence of antibodies in the epidermis of certain fish species is well established, the mechanism of production is still quite obscure. It was thought possible to obtain some information regarding this subject, by stimulating an immune response with a known antigen and subsequently detecting the availability of specific antibodies in the epidermal layer of the skin. A group of fish were immersed in a Vibrio anguillarum vaccine, following the manufacturers instructions and later, attempts were made to detect the existence of anti-V. anguillarum antibodies in the skin samples.

6.1 - MATERIALS AND METHODS

6.1.1 - FISH

12 juvenile rainbow trout (6 males and 6 females), weighing between 70 and 200 grammes (mean weight 110g) were obtained from TRUTURÃO and brought into the Veterinary School of Lisbon, where they were kept in six plastic tanks, in a recirculating water system (figure 1). They were fed on the same commercial pelleted diet used at the farm. The water temperature varied from 11°C in February at the beginning of the experiment, to 14°C in March - April, towards the end.

6.1.2 - VACCINE

A Vibrio anguillarum vaccine from Wellcome Laboratories was employed. The particular batch used, EZ 340, was kindly supplied by Drs. M. Horne and M. Tatner, from the Institute of Aquaculture, University of Stirling.

6.1.3 - VACCINATION METHOD AND SCHEDULE

The vaccine was applied by direct immersion, after diluting it 1: 9 in the water from the tanks (total volume - 6 litres). The fish were netted and individually immersed in the vaccine for 20 seconds. After this, they were reintroduced in their original tanks. The controls were also netted and immersed in a bucket with the same amount of water and for the same length of time. The whole schedule was carried out in the following way:

- after 10 days of adaptation to the new environmental conditions, eight of the fish were vaccinated and two used as controls. Two other fish were killed in order to have 0 day controls;

- two weeks later, two vaccinated and one control were killed and sampled;

- three weeks after the vaccination the remaining fish were again immersed in the Vibrio anguillarum vaccine, exactly as before. The control fish was again immersed in water from the tanks;

- one week after this second vaccination, three of the vaccinated fish were again killed and two weeks after that,the surviving three fish and the control were also killed.

6.1.4 - SAMPLING METHODS

All fish were anaesthetized prior to killing with MS 222. Before death, blood was collected from the dorsal aorta, and samples of head and lateral skin taken.

The blood was allowed to clot at room temperature for two hours (approximately) and at 4°C overnight. The serum was removed after centrifugation at 2500 rpm (20 minutes), and kept at -80°C before use.

Mucus was also obtained by gently scraping the skin with a scalpel blade, avoiding all contact with blood. The volume of mucus obtained was measured and

30% dilutions in saline were made. They were then wholly mixed and centrifuged at 2 500 rpm (20 minutes) to remove occasional scales and suspended cells. The supernatant was collected and kept in the freezer at -80°C.

The skin samples were submitted to the same procedures already described for light and electron microscopical observations (see chapters 1 and 2).

For immunocytochemistry the procedure was the same as already described. The Karnovsky fixative was used with or without osmium post-fixation and the fragments were included in the Epon-Araldite mixture, polymerized at low temperature (see chapter 5).

6.1.5 - PREPARATION OF A RABBIT SERUM ANTI-VIBRIO ANGUILLARUM

A rabbit was injected subcutaneously at several sites along the dorsal surface with a mixture of the vaccine and Freund's complete adjuvant (final volume--3ml/rabbit). The first booster was applied three weeks later. The vaccine was again mixed with adjuvant, but this time using the incomplete form (final volume --3ml/rabbit). The second booster, three weeks after the first one, was performed in the same way, with an extra inoculation of 1 ml of vaccine administered intravenously. The rabbit's blood was collected before the immunization began and one week after each booster. The serum was collected as previously described (see chapter 4) and the presence of agglutinating antibodies was tested.

6.1.6 - DETECTION OF AGGLUTINATING ANTIBODY IN THE SERUM OF THE INOCULATED RABBIT AND IN THE SERUM AND MUCUS OF THE VACCINATED TROUT

Serial doubling dilutions of the inoculated rabbit serum from 1: 2 to 1: 4 096 were made with 0.01 M PBS, in "U" or "V" bottom microtest plates. The final volume in each well was 25 µl. Serum from an uninjected rabbit was used as control. 25 µl of

the vaccine were added to each well. The plates were shaken and left to incubate for one hour at 37°C and overnight at 4°C. The end point was taken as the lowest dilution to show visible bacterial agglutination and its reciprocal taken as the titre.

The presence of agglutinating antibody in the rabbit serum against two other bacteria regularly isolated from trout of the same farm was also determined, in order to evaluate the possibility of cross reactions. The bacteria were Aeromonas hydrophila and Pseudomonas sp. and the bacterial suspensions were kindly supplied by the Bacteriology Department of the National Laboratory of Veterinary Research (L.N.I.V.), Lisbon. The bacterial agglutination technique used was exactly as previously described.

The presence of agglutinating antibodies against Vibrio anguillarum, Aeromonas hydrophila and Pseudomonas sp. in the serum and mucus of the trout used in the vaccination experiment was also tested. The procedure was the same as that used to titrate the inoculated rabbit serum, except for the temperatures of incubation. The plates were incubated at room temperature (±25°C) for two hours and overnight at 4°C.

6.1.7 - ABSORPTION OF THE INOCULATED RABBIT SERUM

In order to avoid nonspecific cross reactions it was found necessary to absorb the rabbit serum with strains of Aeromonas hydrophila and Pseudomonas sp. Each absorption was prepared in the following way: a heavy suspension was made from a fresh 24 hours culture of the bacteria on tryptic soy agar. The total culture of 20 Petri dishes was collected without dilution (±3 cc). Three cc of serum were thoroughly mixed with the culture and left for one hour at 37°C and overnight at 4°C. The following day, the suspension was centrifuged at 14 000 rpm for 30 minutes to remove the bacterial cells. The serum was carefully removed with a Pasteur pipette. Usually, one c.c. of serum was lost during each absorption procedure.

Following the absorption, the sera were newly tested by agglutination to evaluate the efficiency of the procedure.

6.1.8 - PREPARATION OF VIBRIO ANGUILLARUM ANTIGEN. DETECTION OF ANTIBODY BY PASSIVE HAEMAGGLUTINATION

Small samples of vaccine (5 ml) were taken each time and submitted to a 20 minute sonication. They were subsequently ultracentrifuged at 14000 rpm and the supernatant carefully collected.

The presence of antigen in the extract was tested by passive haemagglutination with the serum of the inoculated rabbit. The passive haemagglutination technique of Stavitsky (1954) was used with a few modifications. To control the specificity of the reaction the rabbit serum was tested with uncoated tannic acid treated sheep red blood cells (SRBC) and serum from an uninjected rabbit was tested with antigen coated tannic acid treated SRBC. The antibody titre was registered as the reverse of the lowest dilution to show 50% haemagglutination.

The presence of antibody against the Vibrio anguillarum antigen extract in the serum and mucus of the vaccinated and non-vaccinated trout was also tested by passive haemagglutination. The technique used was the same as that described for the rabbit serum (see appendix for details on the methods).

6.1.9 - IMMUNOENZYMATIC SANDWICH TECHNIQUE TO DETECT ANTI-VIBRIO ANGUILLARUM ANTIBODIES IN THE EPIDERMIS

The method chosen was the sandwich technique developed by Coons, Leduc and Connolly (1955), modified by Sainte-Marie (1962) for paraffin-embedded tissues. However, the present studies utilised resin-embedded skin samples and, in place of the fluorescent labelled antibodies, utilized the unlabelled antibody method of

Sternberger (1974), already referred to as the PAP method (see chapter 5).

Semithin (2 µm) sections of osmicated and non-osmicated resin embedded skin samples of vaccinated and non-vaccinated fish were prepared as explained previously (see 5.1.2.). The resin removal was accomplished in the same way as before with a saturated solution of sodium hydroxide in absolute ethanol.

The sections were subsequently hydrated and washed with the buffer, the same as that used for the PAP light microscopy immunostaining technique: 50 mM Tris(HCl), pH 7.6 in 0.15 M sodium chloride (tris saline). The sections were then exposed to the antigen solution for 20 minutes at room temperature.

For control purposes some sections were overlaid, for the same length of time, with buffer or with tryptic soy broth (TSB) used to culture the V. anguillarum and therefore present in the vaccine (Tatner and Horne, 1983). Rabbit serum anti- V. anguillarum was also used after being absorbed with the vaccine and with TSB to control nonspecific reactions.

The fixation of the antigen was found advantageous, as recommended by Sainte-Marie (1962), and pre-cooled 95% ethanol was used for a period of 30 minutes. After three washings in buffer the sections were incubated with the rabbit serum anti-Vibrio anguillarum, for 90 minutes at room temperature. Several dilutions of the antiserum were tested, from 1: 10 to 1: 800. The serum was diluted in tris saline with 1% normal swine serum. Normal rabbit serum from an uninjected rabbit was used as control, at the same dilutions. 20 µl of mucus from non-vaccinated trout were added to each 300 µl aliquot of the diluted rabbit serum. The objective was to inactivate any possible nonspecific reactions with the complex carbohydrates of the mucus present in the goblet cells in the epidermis. The mucus had been collected as previously explained (6.1.4) and its protein content, after dilution, was 0.140 mg/ml (Gornall colorimetric method for total proteins, Laboratories

Knickerbocker, S.A.E.).

When this incubation was finished, all sections were carefully washed and the swine serum anti-rabbit IgG was overlaid in a 1: 30 dilution in tris saline for 30 minutes. This was followed by new washings and incubation with the peroxidase-antiperoxidase complex, diluted 1: 80 in tris saline, which took 45 minutes. These latter incubations proceeded at room temperature. The sections were again washed in buffer and the peroxidase activity demonstrated by the Graham and Karnovsky method (1966; cf. Sternberger, 1974 and Larsson, 1981). 100 cc of a 0.05% solution of 3,3' - diaminobenzidine tetrahydrochloride (DAB, SIGMA), in tris saline, with 0.01% H_2O_2 was prepared and the slides left to soak for 10 minutes. The developing time was significantly reduced from that used in the immunostaining PAP method (see 5.1.2).

The sections were finally thoroughly washed in distilled water and stained with Erlich's haematoxylin for 10 minutes. Dehydration and mounting were completed as for any routine staining procedure.

6.2 - OBSERVATIONS AND RESULTS

6.2.1 - LIGHT MICROSCOPY

The observation of the skin of the vaccinated immature rainbow trout showed no particular changes regarding its structure, whenever compared with the skin of adults or with the skin of non-vaccinated fish. The fully developed dermis and epidermis exhibited at times a considerable degree of infiltration by small cells with darkly stained nuclei, but this fact was noticeable both in vaccinated and non-vaccinated fish. No quantitative methods were applied to evaluate the amounts of infiltrated cells, but the number of samples studied would not allow the establishment of significant conclusions. No differences were noticed between the fish killed two weeks after the first vaccine exposure and the ones killed three or four weeks after the booster.

6.2.2 - ELECTRON MICROSCOPY

As for the light microscopy, no significant changes were noticed between vaccinated and non-vaccinated rainbow trout juveniles, in terms of skin fine structure. Clusters of lymphocyte-like cells were frequently found, but again with no particular incidence for any of the groups (figure 97).

The only unexpected observation regarding this group of juvenile fish concerned the frequency of apparently multinucleated cells which were found between FCC's and that have been mentioned in chapter 2 (figure 48). However, these cells were present both in vaccinated and non-vaccinated trout.

6.2.3 - TITRATION OF ANTIBODIES

The antibodies titres against Vibrio anguillarum, Aeromonas hydrophila and

Pseudomonas sp. registered in the serum and mucus of vaccinated and non-vaccinated trout are shown in Table I and II respectively. It should be noticed that the mucus had already been diluted (30%) before being tested for the presence of antibody. The titres registered in Table II are for diluted mucus.

The antibody titres against the same bacteria registered in the serum of the inoculated and non-inoculated rabbits are shown in Table III. No antibodies to Vibrio anguillarum were detected in the serum of the inoculated rabbit prior to immunization.

TABLE I - Antibody titres registered in the serum of vaccinated and non-vaccinated trout.

	Vaccinated	Bacterial agglutination			Passive haemagglutination
		Aeromonas hydrophila	Pseudo-monas sp.	Vibrio anguillarum	Vibrio anguillarum
1	Killed 15 days post 1st immersion	N.T.	N.T.	–	8
2		2	3	–	16
3	Killed 8 days post 2nd immersion	4	–	–	N.T.
4		4	–	–	8
5		2	4	–	32
6	Killed 15 days post 2nd immersion	2	16	–	32
7		8	2	–	16
8		N.T.	N.T.	–	N.T.
	Non Vaccinated				
1	Killed on day 0	16	2	–	16
2		–	–	–	–
3	Killed 15 days post 1st immersion	2	N.T.	–	4
4	Killed 15 days post 2nd immersion	4	8	–	16

N.T. - Not Tested

TABLE II - Antibody titres registered in the mucus (30%) of vaccinated and non-vaccinated trout.

	Vaccinated	Bacterial agglutination			Passive haemagglutination
		Aeromonas hydrophila	Pseudo-monas sp.	Vibrio anguillarum	Vibrio anguillarum
1	Killed 15 days post 1st immersion	–	4	–	–
2		–	–	–	–
3	Killed 8 days post 2nd immersion	–	–	–	–
4		–	–	–	2
5		–	–	–	4
6	Killed 15 days post 2nd immersion	–	4	–	4
7		–	4	–	4
8		–	2	–	2
	Non-Vaccinated				
1	Killed on day 0	–	2	–	2
2		N.T.	N.T.	N.T.	N.T.
3	Killed 15 days post 1st immersion	N.T.	N.T.	–	–
4	Killed 15 days post 2nd immersion	–	–	–	4

N.T. - Not Tested

TABLE III - Antiboby titres registered in the serum of inoculated and non-inoculated rabbits.

V. anguillarum	Bacterial agglutination			Passive haemagglutination
Immunized rabbit	V. anguillarum	Aeromonas hydrophila	Pseudo-monas sp.	Vibrio anguillarum
Before absorption	2048	2	4	1248
After absorption	N.T.	0	0	
Non-Immunized rabbit				
Before absorption	0	0	128	0
After absorption	N.T.	N.T.	16	

N.T. - Not Tested

6.2.4 - DETECTION OF ANTI - V. ANGUILLARUM ANTIBODY IN THE EPIDERMIS OF VACCINATED TROUT BY THE SANDWICH TECHNIQUE

The presence of antibody was considered positive whenever dark brown deposits of the polymerized diaminobenzidine were located in the semithin sections.

Antibody against Vibrio anguillarum was therefore detected in the epidermis. However, both vaccinated and non-vaccinated fish showed positive results. In fact, brown deposits were constantly found over the mucous cells, both in skin samples which had not been post-fixed with osmium (figures 98 and 99) and in osmicated specimens (figure 100). The mucous cells stained quite intensely taking a granular brown appearance. In the osmicated tissue, the positive mucous cells showed more like a brownish background stain, with a few outstanding dark-brown granules.

The sections used as control of the method were consistently unstained, both when the antigen layer had been replaced by buffer or by TSB (figure 101) and when the rabbit anti- V. anguillarum serum was replaced by normal rabbit serum (figure 102).

When the incubation proceeded with the rabbit serum anti-V. anguillarum absorbed with the vaccine, the reaction was negative, but still positive when the serum had been absorbed with TSB alone. In Table IV the results of the immunoenzymatic sandwich technique are shown, with the scheme of controls used to avoid nonspecific reactions.

The immunocytochemical PAP method described in chapter 5 to detect antibody--positive cells in semithin sections was equally applied, using the rabbit serum anti-trout IgM. Immunodeposits were again located over the mucous cells of the tested sections.

TABLE IV - Results of the immunoenzymatic sandwich technique applied over skin sections of rainbow trout juveniles in order to detect anti-Vibrio anguillarum antibodies.

	Vibrio (Vaccine)	Tryptic soy broth (TSB)	Without antigen
Serum anti-Vibrio	Positive	Negative	Negative
Serum anti-Vibrio absorbed with TSB	Positive	Negative	–
Serum anti-Vibrio absorbed with the vaccine	Negative	–	–
Normal serum	Negative	Negative	–

6.3 - DISCUSSION

As was reported in the observations, no particular modifications were noticed in the skin of rainbow trout juveniles following the vaccination experiment. However this does not necessarily mean that the vaccine had no effect whatsoever on the integument because it may have produced functional adjustments without inducing morphological changes.

It should be pointed out that multinucleated cells seen in the electron microscopy observations might represent an inflammatory response to stressful environmental conditions affecting fish skin, as the holding tanks were supplied with tap water (dechlorinated) in a recirculating system, not ideal for holding rainbow trout.

No proof was obtained that the fish vaccinated in the present work were really protected, as challenge with virulent strains of V. anguillarum was not performed. However, the vaccine used was a fully tested commercial vaccine and the time of immersion was chosen in agreement with the results of the work of Tatner and Horne (1983), who actually supplied the vaccine. In fact, earlier authors had already determined that a relatively small antigenic mass was able to elicit an immune response (Gould et al. 1978; Nelson et al. 1985). Short immersion times have also been used by Johnson, Flynn and Amend (1982), who successfully immunized salmonids against two bacterial diseases.

The present work has shown that the titration of anti-Vibrio anguillarum antibodies in the serum and mucus of trout by bacterial agglutination is an inaccurate method whenever compared with passive haemagglutination. As low levels of antibody were present, only a sensitive method was likely to detect them. Although the highest titres have been registered in vaccinated fish (see Table I), they were only slightly superior to the titres registered in the non-vaccinated fish.

Low serum antibody titres were frequently reported by authors who used direct immersion or hyperosmotic infiltration methods to immunize fish. Such was the case of Croy and Amend (1977) who introduced hyperosmotic infiltration as a technique for large scale vaccination. Excellent protection against V. anguillarum was achieved in sockeye salmon through this method, but the agglutinin titres were consistently low.

Hyperosmotic infiltration was also confirmed as an excellent method for immunizing fish by Antipa and Amend (1977) who immunized coho salmon and chinook salmon (Oncorhynchus tshawytscha) against V. anguillarum and Aeromonas salmonicida.

However, hyperosmotic infiltration has since been replaced by direct immersion and by spray vaccination as more suitable methods for large scale immunization. Gould et al.(1978) introduced these last two methods as less stressful and producing very good results in coho salmon and rainbow trout, following immunization with V. anguillarum bacterin. The antibody titres registered by these authors were consistently low, although no mortality occurred upon challenge.

Good protection against V. anguillarum was subsequently obtained by several authors using direct immersion or bath vaccination to immunize salmonids, although no antibody titres were measured (Egidius and Andersen, 1979; Gould, Antipa and Amend 1979; Antipa, Gould and Amend, 1980 ; Nelson et al. 1985).

Baudin-Laurencin and Tangtrongpirus (1979) working on vaccination of coho salmon and rainbow trout in Brittany, concluded that protection against V. anguillarum remained satisfactory, even when the agglutinin levels had decreased.

The question of low antibody titres in protected fish was again raised by Sakai et al. (1984) who reported to have found equivalent titres of circulating antibodies

against V. anguillarum in both vaccinated and non-vaccinated rainbow trout. The immunization method used by these authors was direct immersion in a bacterin.

Identical results were obtained by Aoki, Sakai and Takahashi (1984) and by Kawano, Aoki and Kitao (1984) in ayu (Plecoglossus altivelis) vaccinated by immersion, with no agglutinating antibodies being detectable in the serum of the vaccinated fish.

In a study on the factors influencing the efficacy of vaccines against vibriosis, Ward, Tatner and Horne (1985) reported that circulating antibody response was not necessary for protection. Thereupon these authors suggested that if circulating antibody was only part of the protective mechanism against vibriosis, then other aspects of the immune response must be relevant, namely locally produced antibody.

The existence of a local immune system in fish was also considered by Lamers and De Haas (1985) who, working in bath vaccination of carp, showed that immunological memory could only be stimulated if the route of challenge had been the same as the priming route.

The hypothesis of a local immune system is in accordance with a local mechanism of antigen uptake. The early work of Gould et al. (1978) on spray vaccination provided some useful information on this subject. They showed that pressure was not required to induce an immune response. Evelyn and Ketcheson (1979) developed a series of studies on the immunogenicity of intact cells and spent culture broth derived from a V. anguillarum culture. They concluded that the soluble immunogens must be large molecules (over 100,000 MW) because they were retained in ultrafiltrates. Following these findings, they suggested that an active uptake of antigen was certainly taking place, because simple diffusion could not explain the entry into fish of such large molecules.

In earlier chapters of this thesis it was shown that cells with characteristics which are typical of macrophages were frequently found in the epidermis, where they could play a role in phagocytosis of foreign molecules. The malpighian cells also appear capable of incorporating foreign material such as melanin granules. In recent work on antigen uptake mechanisms in rainbow trout, antigen was found within epithelial cells six hours after spray vaccination (Hockney, 1985).

As the skin, namely the epidermis, appears to be fully equipped for antigen trapping, it seemed reasonable to question whether antibodies could also be locally produced, having a direct effect on the fish body surface.

The sandwich technique that was applied to skin sections of vaccinated trout in the present study appears to demonstrate the presence of specific antibody in the mucous cells in the epidermis.

The sandwich technique for histochemical demonstration of specific antibody was initially developed by Coons, Leduc and Connolly (1955), who proved that plasma cells were the major site of antibody formation. They used frozen sections of rabbit organs and immunofluorescence methods. Their work was later confirmed by Sainte-Marie (1962) on sections of paraffin-embedded tissues, again using immunofluorescent labelling methods.

In the present thesis the instructions of Sainte-Marie (1962) were carefully followed, with the necessary adaptations for plastic-embedded tissues. The controls were used to remove the possibility of nonspecific reactions giving false positive results.

The antigen extract applied in the sandwich technique, obtained after sonication and ultracentrifugation of the vaccine, although not incorporating whole bacteria or even their fragments, contained large amounts of immunogens.

as was shown by the strong precipitation reaction it formed with the rabbit serum anti-V. anguillarum. The spent broth of the vaccine used in the present work was certainly rich in soluble immunogens, similar to that which was referred to by Evelyn and Ketcheson (1979). The positive results of the sandwich technique obtained with the rabbit serum anti-V.anguillarum absorbed with tryptic soy broth (TSB), showed that the immuno-precipitates formed had no relation to antibodies anti-TSB which could be present in the rabbit serum.

The possibility of the results of the immuno-sandwich technique being artefactual is therefore rather remote and makes it possible to suggest that anti-V. anguillarum antibodies were present in the epidermal mucous cells of the rainbow trout in this study.

However, the positive reaction obtained in the same cells of fish which were not vaccinated rules out the possibility of relating the presence of antibodies only with the immunization procedure.

It could be argued that the rabbit serum anti-V. anguillarum was, in fact, recognizing other bacteria or bacterial antigens, which could be present in the epidermal sections due to previous infections with Aeromonas or Pseudomonas. But, to avoid such eventuality, the rabbit serum anti-V. anguillarum was absorbed with these bacteria prior to use in the immunosandwich technique.

One explanation for the presence of antibody in vaccinated and non-vaccinated fish could be that, although no previous history exists of Vibrio anguillarum infections in the farm from which the fish were obtained, a strict control of the presence of this type of bacteria was never made.

The trout used in this vaccination experiment were not raised in a controlled environment, so they could have had an early contact with bacteria sharing some

of the V. anguillarum antigenic determinants.

A number of facts remain unexplained regarding the fish immune response to V. anguillarum. For example, immunization of trout against this bacterium seems to increase the antibody titres versus other microbial agents such as Yersinia ruckeri (Tatner, 1986, personal communication).

The presence of antibody in the mucous cells was previously shown by the indirect immunocytochemical technique described in chapter 5. But, as was reported in the observations, not all mucous cells reacted positively, suggesting individual differences between these cells, even in the same limited epidermal area. Differences in the mucus composition were also suggested in the discussion of the light and electron microscopy results due to the variable staining characteristics of the mucous vesicles.

Although immunoglobulins in the mucous cells may be considered an unexpected result, the presence of antibody in the outer skin covering mucus has been shown for a variety of species, as was referred to in the introduction (Fletcher and Grant, 1969; Di Conza, 1970; Bradshaw et al. 1971; Fletcher and White, 1973; Ourth, 1980; St.Louis-Cormier et al. 1984).

The titres obtained in the mucus were rather low, but this might be expected when one takes into account the distribution of mucous cells in the epidermis and the way these cells mature and reach the surface. Although a large number of immunoglobulin-containing mucous cells was shown in the epidermal sections, only a very reduced number of these cells was actually capable of discharging its own secretion into the surface. Therefore, only small amounts of antibody are probably present at any one time. It may well be that the small amounts of active superficial immunoglobulins are more than enough to protect the skin against most pathogens.

It is not possible to conclude from the results of the present work whether the antibodies in the mucous cells were locally produced, nor if any stimulation occurred after the vaccination, but the involvement of mucous cells in a skin secretory system, already suggested by several authors, deserves further consideration.

GENERAL DISCUSSION

The present investigation was directed from the start towards a better understanding of the skin's importance in defense mechanisms.

It was shown through light microscopical observations how the skin, and especially the epidermis, is such a plastic organ, changed its structure during the growing phase, and during adult life according to sexual maturation and under hormonal influence. Although research work concerning seasonal changes is more advanced in salmonids, similar findings are being reported by authors studying other species. Such is the case of Burton and Fletcher (1983) who found profound cyclical changes in the epidermal thickness of the winter flounder Pseudopleuronectes americanus. Sexual dimorphism was also displayed by this fish species, the males developing a significantly thicker epidermis than the females, during the spawning period.

The variability shown by the skin was even more evident in the electron microscopical observations. Considering that the basis of the epidermal tissue is the filament-containing cells and the mucous cells, both of identical embryonic origin, it seems more likely that other cells found in this tissue are, in fact, entering from other tissues. Assuming, as was shown in the present study, that these intruding cells are mainly lymphocytes and macrophages, it appears that the epidermis of trout is able to locally express most, if not all, immunological functions.

Antigen uptake mechanisms have been studied in mammalian epidermis. Langerhans cells have long been known to be antigen trapping cells (Shelley and Juhlin, 1976), which play an important role in allergic contact dermatitis (Silberberg, Baer and Rosenthal, 1974), capable of antigen presentation (Breathen and Thorsby, 1980), and functioning as stimulatory of cytotoxic T lymphocytes

(Tsuchida, Iijima, Fujiwara, Pehamberger, Shearer and Katz, 1984).

Typical Langerhans cells were not found in the epidermis of trout in the present study. Structures which resembled the typical Birbeck granules of these cells were reported by Mittal et al. (1980) in an epidermal cell of Monopterus cuchia, but this was the only reference to Langerhans cells in fish found in the literature.

However, other cells exist in the human epidermis which share many features with the Langerhans cells, except for the presence of the typical granules. These are the so called indeterminate cells, or agranular dendritic cells, which together with the Langerhans cells and the melanocytes are the three forms of epidermal dendritic cells (Zelickson and Mottaz, 1968). The two former cell types share antigenic determinants as was demonstrated through immunoelectronmicroscopic studies by Rowden, Phillips and Lewis (1979) and by Faure, Schmitt, Dezutter-Dambuyant, Frappaz, Gaucherand and Thivolet (1984). This fact led Rowden et al. (1979) to suggest that indeterminate cells could be dermal macrophages which migrated into the epidermis to replace emigrated Langerhans cells. A similar hypothesis was advanced by Hoefsmit , Duijvestijn and Kamperdijk (1982) to explain the development of monocytes into epidermal Langerhans cells. These would subsequently originate other cells found in the different lymphatics and in the thymic dependent areas of peripheral lymphoid organs or in the thymus medulla, i.e. the so called veiled cells and the interdigitating cells respectively. According to these authors, they all shared the same surface determinants as well as the typical Birbeck granules, whose formation in the Langerhans cells was thought to be induced by a particular epithelial micro-environment, present in the epidermis and in the thymus.

The migration of macrophages into the epidermis of trout could be the equivalent to what seems to occur in the human skin and the lack of a specific micro-environment may prevent their further development into Langerhans cells.

A recent report by Wood, Willoughby and Beakes, (1986) on infection by Saprolegnia parasitica in brown trout, revealed that fungal mycelium derived from challenge zoospores which had grown on the fish mucus for 24 hours had mononucleated cells attached to it that could only be originally from the fish skin.

They interpreted these cells as being lymphocytes or neutrophils but a more exact identification was not made. In fact, these cells could also be macrophages and still compatible with the authors explanation for their occurrence, which was the existence of a cell-mediated defense mechanism. In a particular case described in the present study, large numbers of what seemed to be monocyte cells were present in the epidermis . These monocytes' ultrastructural characteristics closely resembled the Langerhans cells described by some authors (Zelickson, 1965; Hashimoto and Tarnowski, 1968), except for the presence of the typical granules.

Another interesting finding of the present study was the traffic of melanin and melanin-bearing macrophages in the skin, which has seldom been reported (Percy, 1970; Roberts et al. 1971) and never in salmonids. Both in adult and juvenile fish, macrophages laden with melanin granules were seen in the dermis, in the epidermis and crossing the basement membrane. These cells may well be related to the melano-macrophages described in many species of fish (Agius, 1980), which appear randomly distributed in the spleen and kidney in salmonids, but show up as aggregated in the so called melano-macrophage centres in most fish species (for review see Agius, 1985). The origin of these macrophages,as well as of the melanin they contain, is still obscure, but they appear constantly related with defense mechanisms, such as phagocytosis of injected particulate material (Ellis, Munroe and Roberts, 1976; Herraez and Zapata, 1986) and antigen-trapping (Ellis, 1980; Alexander et al. 1982; Lamers and Pilarczyk, 1982). In a variety of advanced bony species the melano-macrophage centres, especially in the spleen, establish close contacts with lymphoid cells which surround them (Agius, 1980) and earlier work on the plaice carried out by Ellis and de Sousa (1974) had already shown the intimate

association between these two types of cells. These authors have shown through autoradiographic studies that circulating lymphocytes of the plaice, labelled with tritiated uridine and reinjected, contained a population of cells which selectively migrated to the lymphoid tissues, reaching the melano-macrophage centres 24 hours after inoculation.

Following these experiences, several investigators suggested that these melanin-containing macrophages could act, either aggregated or dispersed in the fish haemopoietic organs, as antigen presenting cells, capable of stimulating lymphocytes (Ellis et al. 1976; Agius, 1980). More recently, their involvement in specific immune reactions was shown by Herraez and Zapata (1986) in the gold fish. These authors reported that primary and secondary immunization with sheep erythrocytes produced a notable increase in the number of cells of the melano-macrophage centres.

Although the concept of involvement of melano-macrophages in immune reactions is becoming less controversial, the presence of melanin within these cells remains rather obscure, in terms of its origin and its possible role.

Melano-macrophages in the kidney and spleen of plaice were DOPA negative (Ellis, 1974 cited by Agius, 1980), precluding the hypothesis of local synthesis of the pigment. Agius (1985) suggested that melanin within the melano-macrophages might be skin derived pigment, produced in melanocytes and later phagocytosed by the former cells, in a process similar to that occurring in higher animals. If this hypothesis is true, it could mean that, prior to being allocated to the haemopoietic tissues, these macrophages had already been established in the skin for a certain period of time, during which they might have come into contact with a variety of antigens, becoming sensitized cells. They could even have had the opportunity of migrating towards the epidermis and again back into the dermis, having a final destination in the kidney or spleen.

Although these considerations seem rather speculative, the fact is that in mammals, as was previously referred to, traffic of cells between dermis and epidermis is known to occur, involving macrophages and Langerhans cells. These cells are also known to migrate towards the regional lymph nodes in allergic contact sensitivity, probably as a circulating population capable of sensitizing lymphocytes (Silberberg-Sinakin, Gigli, Baer and Thorbeck, 1980) The comparison between Langerhans cells and melano-macrophages is therefore valid.

The role played by the pigment itself is not clearly understood. It may be that no particular function is accomplished by the melanin, its presence within macrophages being a mere accident due to phagocytosis. If the cells were incapable of destroying the pigment, they would accumulate it together with other substances they are known to store such as lipofuscin and iron (Agius, 1979; Agius and Agbede, 1984).

Keratinocytes in the human skin have melanin granules within melanosome complexes, which in fact are known to be lysosomes with acid phosphatase activity (Hori et al. 1968). It seems that the darker pigmentation of the skin of negroids could be due to larger melanosomes which accumulate within the keratinocytes, due to lack of specific lysosomal activity which exists in caucasoids (Mitchell, 1968, cited by Ghadially, 1982).

Melanin is a substance with an extremely high resistance to oxidizing agents and its presence within macrophages could be due to a lack of certain lysosomal enzymes capable of destroying it. In fact, no intermediate stages of destruction of melanin were found in the macrophages located both in the dermis or epidermis of rainbow trout. As far as it was possible to appreciate through the literature review, the same applies to melano-macrophages described by other authors in internal organs.

Further research on the role of these cells seems necessary, as evidence accumulates that they must respond to certain pathological conditions, especially in inflammatory reactions due to the presence of parasites (Mawdesley-Thomas and Young, 1967; McQueen, MacKenzie, Roberts and Young, 1973; Cruz e Silva and Freitas, 1984). Migration of pigment-containing macrophages was also described in wound healing studies by Anderson and Roberts (1975).

Other controversial findings of the present thesis seem to be the presence of antibody in the epidermal mucous cells, although, as was noted previously in chapter 5, several authors have already reported the existence of immunoglobulins (Igs) in the cutaneous mucus in a variety of fish species. They have also been identified in the intestinal mucus of plaice (Fletcher and White, 1973).

Several possibilities could be advanced to explain the presence of Igs in the cutaneous mucus. First, diffusion of serum Ig into the mucus following parenteral or oral immunization. Second, diffusion of dermally produced Ig into the mucus, following parenteral, oral or local immunization and third, production of Ig in the epidermis itself, following local antigenic stimulation. An interesting observation is that none of these possibilities precludes the existence of the others. And, furthermore, they do not rule out other alternatives, such as cell-mediated immune responses.

More evidence is accumulating that the antibodies in the mucus, although antigenically identical to the serum immunoglobulins, may differ in other aspects. Lobb and Clem (1981 b) identified three types of immunoglobulins in the mucus of the sheepshead, one type of tetrameric high molecular weight Ig, identical to the serum counterpart and two types of low molecular weight dimeric Ig. Although these authors have shown through administration of radioiodinated serum Ig, that no transudation or active transport occurred into the mucus or bile, they did not work with antigenically stimulated animals, and the time which elapsed between

the administration of the radioiodinated Ig and the collection of mucus and bile was not indicated. The existence of three different types of immunoglobulins seems to suggest three distinct ways of producing them, systemically or locally. As a particular protein component was identified linked to one of the dimeric Igs, the existence of an equivalent of the mammalian secretory component was suggested. On the other hand, the work of Fletcher and White (1973) in plaice has shown that cutaneous mucus only had detectable antibodies to V. anguillarum in fish which had been parenterally immunized and whose serum antibody titres were high.

Local production in response to local immunization has not been frequently tested. Again the work of Fletcher and White (1973) has shown that the immunoglobulin production obtained in the intestinal mucus after oral and therefore local, immunization, was higher than in the serum.

In mammalian intestine several types of immunoglobulin producing cells have been identified located in the lamina propria (Brown and Bourne, 1976; Hart, 1979; Willard and Leid, 1981). All cited authors reported the presence of high numbers of IgA producing cells and they all detected IgA in the apical cytoplasm and luminal surface of epithelial crypt cells. Brown and Bourne (1976) considered IgM to have a secretion pathway similar to IgA, for it was found with the same location, although in lower amounts.

This distribution of immunoglobulins in the mammalian intestinal juice is similar to the immunoglobulin profile found in the milk, in which locally produced IgA predominates (Bourne, 1976). The stimulus for mammary IgA production has long been known to be mostly due to oral immunization (Heremans and Bazin, 1971; Goldblum, Ahlstedt, Carlsson, Hanson, Jodal, Lidin-Janson and Sohl-Akerlund, 1975). This relathionship between intestine and mammary gland indicates a traffic of sensitized lymphoid cells or plasma cells, from the areas were they were stimulated (intestinal mucosa, Peyer's patches, mesenteric lymph nodes) into sites where

antibody production is necessary.

Studies of this kind were not carried out in fish and unfortunately the previously mentioned studies of Ellis and de Sousa (1974) on migration of labelled lymphocytes to selective areas, did not include the skin.

Studies involving the location of immunoglobulins in the skin of cattle and sheep (Lloyd, Jenkinson and Mabon, 1979) and in the skin of dog (Garthwaite, Lloyd and Thomsett, 1983) showed a similar distribution of IgG, IgM and IgA in all three species. IgA was generally found in the sweat gland cells and excretory duct, demonstrating the function of these glands in the secretion of this immunoglobulin. IgG and IgM were, however, absent from the epidermis, particularly in the dog (Garthwaite et al. 1983), although they were consistently found in the interstitial tissue throughout the dermis. Therefore, it is still unknown how these Igs reach the surface, because they have always been identified in skin surface washings of the above referred to animals (Jenkinson, Lloyd and Mabon, 1976, 1979).

An interesting parallel could be drawn between the secretory role played by the sweat glands in the production of surface IgA in mammals and an equivalent role which could be played by the unicellular mucous cells in the fish skin.

Mature brown trout males are known to be very susceptible to infections with fungus (Richards and Pickering, 1978) and parasites (Pickering and Christie, 1980) during the sexual maturation period. This susceptibility of breeding fish was also noticed in other species of salmonids, namely in the Pacific salmon by Smirnov (1959). It is common knowledge amongst rainbow trout farmers that sexually precocious males display severe skin lesions similar to the ones occurring in mature males during the spawning period. Amongst the generally described changes produced in the males' skin during this period is the decrease in the

number of mucous cells (Pickering, 1977; Peleteiro, 1981; Pottinger and Pickering, 1985 a). Assuming that such cells play an important role in the secretion of mucus antibodies it seems possible to relate both facts and therefore to suggest that the higher skin susceptibility may be related to a decrease in available immunoglobulins in the mucus, although the direct protective effects of surface mucus,in preventing hydration of surface cells and removal of infectious agents by continual sloughing,must be considered.

The influence of hormones in the skin structure has been studied by several authors(McBride and Van Overbeeke, 1971; Yamazaki, 1972 ; Pottinger and Pickering, 1985 a,b) . Hormones also seem to influence the secretory immune system as in the case of the eye of rats which was studied by Sullivan, Bloch and Allansmith (1984). These authors concluded that testosterone induced an increase in the in vitro production of secretory component by the exorbital glands. Such findings are not in contradiction with what was previously suggested about hormonal influence in the production of mucus, because the work of Pottinger and Pickering (1985 b) has shown that testosterone had no effect on superficial mucous cells, the decrease in their number being under the influence of 11-ketotestosterone which is the dominant androgen in male salmonids. This latter hormone may in fact exert an opposite effect to maturation of mucous cells, preventing them reaching the surface of the skin and therefore restraining the secretion of antibody as well as of other mucous components that interfere in local defense mechanisms.

The mucus of fish is deserving the ever growing attention of researchers, interested in understanding the various defence mechanisms of this group of vertebrates (for review see Ingram, 1980). C-reactive protein was detected in the mucus of tilapia (Sarotherodon mossambicus) by Ramos and Smith(1978),following the induction of tissue inflammation. Traces of lysozyme activity have been identified long ago in the skin of plaice by Fletcher and Grant (1968) and Murray and Fletcher (1976). Their findings were later confirmed by Hjelmeland, Christie

and Raa (1983) in rainbow trout, salmon, char, plaice and other species. They also identified haemagglutinins and a trypsin-like protein. This trypsin was found to cause dramatic changes in the wall structure of V. anguillarum, thus rendering the bacterium more susceptible to other antimicrobial agents. These authors also noticed that the mucus trypsin increased the levels of soluble antigens in growing culture of the same microorganisms, which led them to suggest that the essential function of the enzyme could be to remove antigenic fragments and facilitate the permeation of antigens into the body through the skin.

Other factors that can influence skin permeability have also been identified in the mucus. Such is the the case of calmodulin, a calcium-dependent activator of various enzymes in eukaryotic cells, which has been purified from the skin mucus of rainbow trout, catfish (Clarias lazera) and tilapia (Sarotherodon mossambicus) by Flick, VanRijs and Bonga (1984). These authors suggested that calmodulin was secreted by the skin goblet cells.

But the malpighian epidermal cells also seem capable of producing substances which interfere in skin defence mechanisms. Some authors have identified a factor produced by carp epidermal cells in vitro, with functional similarities to mammalian interleukin -1 (Sigel, Hamby and Huggins, 1986). This factor plays multiple roles in inflammatory and immune reactions and its identification in fish provides evidence for the early phylogenetic origin of these substances.

Citing Billingham and Silvers (1971) "the skin is the largest organ in the body with a remarkable protean structure. It presents a tremendous range of regional structural and functional adaptations and fulfills a variety of physiological functions that far surpass those of any other organ - in this sense it must be regarded as a super-organ, made up of sub-organ systems". Although these authors were referring to the human skin it seems that the same concept can be fully applied to fish.

The skin of fish must display a high range of defence mechanisms which allow these animals to survive in hostile environment, very often under the influence of toxic substances, parasites and microorganisms. As more is learned about the skin, its cellular composition and its secretions, the easier will it be to develop better ways to protect fish from disease agents. Vaccination seems to be the ideal way to take full advantage of the skin potentialities by stimulating all types of specific and even nonspecific defence systems and is already proving very helpful in combatting fish diseases.

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APPENDIX

FIXATIVES USED THROUGHOUT THIS STUDY

1) Phospate buffered formalin (light microscopy).

Formaldehyde solution (37%) -100 ml; anhydrous di-sodium hydrogen phosphate (Na_2HPO_4) - 6.5 g; sodium dihydrogen phosphate mono-hydrate ($NaH_2PO_4H_20$) - 4 g; distilled water - up to 1000 ml.

2) Karnovsky fixative mixture (Karnovsky, 1965)

Solution A

Paraformaldehyde - 2g

Double distilled water - 50 ml

This solution was dissolved by gently heating, in a water-bath (no more than 60 - 70°C), with agitation. 1 to 3 drops of sodium hydroxide (1N) were added to clarify the solution.

Once cooled, 5 ml of 50% gluteraldehyde were also added.

Solution B - 0.2 M Sodium cacodylate

Sodium cacodylate - 4.28 g

Double distilled water - up to 100 ml

Solution A was made up to 100 ml with solution B. The pH was adjusted to 7.4 with 0.1N hydrochloric acid.

25 mg of calcium chloride were added to the final solution.

3) Fixative used for gold labelling immunocytochemistry

4% paraformaldehyde;

0.2% picric acid;

in 0.1 M cacodylate buffer, containing 5 mM calcium chloride.The pH was adjusted to 7.4 with 1N hydrochloric acid.

BUFFERS:

1) For light and electron microscopy immunocytochemistry:

50 mM Tris (hydroxymethyl) aminoethan, MERK, (M.W. - 121.14), pH 7.6, in 0.15M sodium chloride.

a) 0.5 M Tris (HCl) stock solution (1 litre), pH 7.6 :

to 60.57 g Tris was added 500 ml of double distilled water;

1 N hydrochloric acid (HCl) was added to adjust the pH to 7.6 (approximately 376 ml). This was then made up to 1000 ml with double distilled water.

b) 0.15 M NaCl (1 litre): 8.77g NaCl in 1 000 ml of double distilled water.

c) Final solution:

to 100 ml of 0.5M Tris(HCl), 900 ml of 0.15M NaCl were added and the final pH adjusted to 7.6 with 1N HCl.

2) For electron microscopy immunocytochemistry:

20 mM Tris(HCl), pH 8.3 with 0.1% Bovine Serum Albumin (BSA), and 0.5 M sodium chloride.

a) Tris (HCl) stock solution (0.2 M), pH 8.3:

to make 100 ml of buffer, 2.43g of Tris was added to 50 ml of double distilled water. The pH was brought to 8.3 with 1N HCl.

The final solution was made up to 100 ml with double distilled water.

b) 0.5M NaCl (100 ml): 2.92 g of NaCl in 100 ml of double distilled water.

c) Final solution:

to 10 ml of 0.2 M Tris(HCl) was added 90 ml of 0.5M NaCl and 1 mg/ml of BSA (Sigma).

Adjustment of final pH to 8.3 was carried out with 1N HCl (only a few drops).

HISTOLOGICAL PROCESSING SCHEDULE

50% ETHANOL - 2 hours;

80% ETHANOL - 2 hours;

95% ETHANOL - 2 x 3 hours;

95% ETHANOL - 2 hours;

ABSOLUTE ETHANOL - 2 hours;

ABSOLUTE ETHANOL - one hour;

CHLOROFORM - 2 x one hour;

PARAFFIN WAX - 3 x 2 hours.

ELECTRON MICROSCOPY PROCESSING SCHEDULE

1) Fixation for two hours in the Karnovsky mixture, at 4°C;

2) 3 x 10 minutes washings in 0.1 M cacodylate buffer, pH 7.4;

3) post-fixation in 1% OsO_4 in 0.1M cacodylate buffer, pH 7.4, for two hours at 4°C;

4) 3 x 10 minutes washings in sodium acetate - acetic acid buffer, pH 5.0;

5) block staining in 1% uranyl acetate in sodium acetate- acetic acid buffer, pH 5.0,for one hour at 4°C;

6) 3 x 10 minutes washings in sodium acetate - acetic acid buffer, pH 5.0;

7) 10 minutes 70% ethanol (occasionally was left overnight);

8) 2 x 10 minutes 95% ethanol;

9) 3 x 10 minutes absolute ethanol;

10) 2 x 15 minutes propylene oxide;

11) 2 x 15 minutes in a mixture propylene oxide: resin (1: 1);

12) 3 x 10 minutes changes of pure resin. The last change was left in the dessicator under vacuum, overnight at room temperature;

13) polymerization was allowed to proceed for two days (48 hours) at 70°C, in gelatine capsules or rubber moulds.

PREPARATION OF THE EPON-ARALDITE MIXTURE FOR ELECTRON MICROSCOPY

EPON 812 or LX 112	17.28 g
ARALDITE	10.52 g
DODECENYL SUCCINIC ANHYDRIDE (DDSA)	32.92 g
DIBUTYL PHTHALATE (DBP, External Plasticizer)	2.00 g
TRI (Dimethyl Amino Methyl) Phenol (DMP 30)	1.20 g
TOTAL	63.92 g

LIGHT MICROSCOPICAL STAINING OF EPON-ARALDITE EMBEDDED MATERIAL WITH THE PEROXIDASE-ANTIPEROXIDASE (PAP) METHOD.

The instructions of Larsson (1981) were carefully followed. For most steps of the method, whenever the slides had to be immersed on a solution, a 10 slide container was used to hold them vertically. For serum incubation the slides were disposed horizontally in large Petri dishes, on top of U shaped glass tubes, in order to keep them separated from the wet filter paper covering the bottom of the dish. This device was necessary to maintain a moist atmosphere for a long period of time.

The method employed the following steps:

1) sodium hydroxide saturated solution in absolute ethanol, for 10 minutes to remove the resin;

2) four washings in absolute ethanol and one washing in 70% ethanol;

3) three washings in double distilled water (from this step onwards, the sections must not be allowed to dry);

4) one 5 minute washing in buffer (50 mM Tris(HCl), pH 7.6 in 0.15 M sodium chloride);

5) twenty minutes washing in 100 ml of buffer with 0.3 ml of 30% hydrogen peroxide (H_2O_2) in order to inhibit endogenous peroxidase (this step was later considered unnecessary);

6) one washing in buffer (5 minutes);

7) the sections were exposed to an inert serum (swine serum 1: 9 dilution in buffer) for 30 minutes, followed by a very quick rinse with buffer (this step was omitted in some of the sections in order to test its significance in the final results and was later considered unnecessary);

8) the rabbit serum anti-trout IgM was applied at the following dilutions: 1: 10, 1:50, 1: 100, 1: 200, 1: 400, 1: 800, 1: 1 600 and 1: 3 200. The incubation time took 20 hours at 4°C with an extra 2 hours at room temperature. The controls were incubated with normal rabbit serum at the same dilutions or with absorbed rabbit serum anti-trout IgM, during the same period of time;

9) three 10 minutes washings in buffer;

10) incubation for 45 minutes (r.t.) in a 1: 30 dilution of the link serum, in the present case, swine serum anti-rabbit IgG;

11) three 10 minutes washings in buffer;

12) the peroxidase-antiperoxidase complex was applied, diluted 1: 80, for one hour;

13) three 10 minutes washings in buffer;

14) peroxidase activity was demonstrated incubating the sections for 30 minutes in a freshly prepared and filtered 0.05% solution of diaminobenzidine, with 0.01% of H_2O_2 (Graham and Karnovsky, 1966; Sternberger, 1974);

15) the slides were subsequently rinsed ten times in flowing tap water and finally in distilled water;

16) staining with Erlich's haematoxylin for 10 minutes, dehydrating in ethanol and xylene and mounting, as in any routine staining procedure, were the final steps of the method.

ELECTRON MICROSCOPICAL STAINING OF EPON-ARALDITE RESIN-EMBEDDED MATERIAL WITH THE PAP METHOD

The instructions of Larsson(1981) and Sternberger(1974) were carefully followed except for the diaminobenzidine (DAB) incubation: a low and high concentration were used. The grids were always floated sections down, on droplets of the different reagents, and washed by rinsing them individually, held by a forceps, delivering the washing buffer as a stream with a Pasteur pipette. The buffer used was 50 mM tris saline, pH 7.6, both for washing and for dilution of the various sera used, as well as for preparing the DAB solution. The grids were submitted to the following treatment:

1) etching in 10% H_2O_2 for 5 minutes. Osmicated specimens were etched for 20 minutes;

2) washing with double distilled water and with buffer;

3) incubation on 10% normal swine serum, for 30 minutes, at room temperature, to block nonspecific protein reactions. Blotting on filter paper and transfer without washing to the next serum;

4) incubation on the rabbit serum anti-trout IgM diluted 1: 400 in buffer, for 20 hours at 4°C, with a further 2 hours period at room temperature (r.t.). The controls were incubated in the same dilution of normal rabbit serum;

5) washing with buffer;

6) blocking steps as in 3);

7) incubation on swine serum anti-rabbit IgG, diluted 1: 30, for 30 minutes (r.t.);

8) washing with buffer;

9) blocking step as in 3) and 6);

10) incubation on PAP, diluted 1: 50 with 1% normal swine serum, for 10 minutes (r.t.);

11) washing with buffer;

12) developing reaction with a freshly prepared solution of DAB (0.0125%) in tris saline, to which H_2O_2 was added just before use, in a concentration of 0.0025%. A

more concentrated solution of 0.05% DAB with 0.01% hydrogen peroxide was alternatively used. The solution was introduced in small containers and the grids left to float for three minutes. If the grids were nickel ones they were kept under agitation on a magnetic stirrer;

13) thorough wash with double distilled water and, finally, the grids were allowed to dry;

14) the DAB polymeres were fixed with 4% osmium tetroxide, for 20 minutes, and washed with double distilled water;

15) no staining was performed in order to avoid misinterpretation of the results.

ELECTRON MICROSCOPICAL IMMUNOGOLD LABELLING METHOD

The grids were floated, sections down, on drops of the reagents displayed on a sheet of parafilm. Whenever more than 4 grids were being handled (up to a limit of 8), microtest plates were used,the small wells being able to firmly hold drops of the reagents, with no risk of cross-contamination. The buffer used was 0.1% BSA-Tris saline, pH 8.3.

For the Epon-Araldite embedded material the following pre-treatment was carried out:

1) etching with a saturated aqueous solution of sodium metaperiodate (60 minutes, r.t.) or with 10% H_2O_2 (5 minutes for non-osmicated specimens), wash with double distilled water (careful jetwash, plus 10 minutes on drop);

2) protease treatment with 0.005 mg/ml in PBS, pH 7.4, for 3 hours at 37°C, followed by a careful wash with PBS (jetwash) and in double distilled water (3 x5 minutes). Some of the grids were not submitted to this step.

From here on the procedure was the same for the Epon-Araldite, Lowicryl or LR White embedded material :

3) wash on double distilled water (5 minutes);

4) incubation on 5% normal goat serum in 0.1% BSA - Tris saline, to inhibit nonspecific protein reactions. The grids were transfered to the next serum without washing. They were only blotted on filter paper;

5) incubation on the rabbit serum anti-trout IgM, diluted 1: 400 in 0.1% BSA-Tris saline, supplemented with 1% normal goat serum, for two hours (r.t.);

6) wash on buffer (3 × 5 minutes);

7) incubation on the gold labelled goat serum anti-rabbit IgG, diluted 1: 25, for one hour (r.t.);

8) wash on buffer (3 × 5 minutes);

9) wash on double distilled water (2 × 5 minutes);

10) contrast with 2% uranyl acetate (45 minutes at 60°C) and lead citrate (5 minutes).

PASSIVE HAEMAGGLUTINATION

1) The sheep red blood cells (SRBC), collected in Alsever solution, were washed with saline and centrifuged at 2000 rpm for 10 minutes. The procedure was repeated three times;

2) one ml of packed cells were diluted in 40 ml of PBS (0.15 M), pH 7.2 (2.5% dilution);

3) 40 ml of diluted red blood cells plus 40 ml of 1/20 000 solution of tannic acid in PBS, pH 7.2, were incubated in a 37°C water bath for 10 minutes. The cells were then gently centrifuged and washed with PBS, pH 7.2. They were finally resuspended in PBS (pH 7.2) at the same dilution as before (2.5%);

4) 4 ml of PBS (0.15 M) pH 6.4, plus 1 ml of Vibrio anguillarum antigen extract (see 6.1.3), plus 1 ml of tannic acid coated cells were mixed in this order, in a small bottle. After gently mixing, the bottle was incubated at 37°C for 30 minutes in a water bath. The cells were finally centrifuged and washed in 2 ml of 1/100 normal rabbit serum (the serum had been absorbed for 10 minutes with an equal volume of

washed, packed SRBC and diluted with saline), used as a stabilizer to balance out the agglutinating effect of the tannic acid. The final cell suspension was made in 1 ml of 1/100 normal rabbit serum;

5) In a microtest plate two fold dilutions of the sera or mucus were prepared, from 1: 2 to 1: 4 096 with 1/100 normal rabbit serum (absorbed with SRBC) in saline. 25 µl of the sensitized SRBC were added to each well. The same volume of non-sensitized tannic acid cells was also used in one row, to test the specificity of the reaction. The plate was shaken and left at room temperature. Two hours later the reaction was read and the degree of haemagglutination registered.

NOTE: All rabbit sera were submitted to a 30 minutes bath at 56°C prior to use, to inactivate the complement. The trout serum and mucus were also submitted to a water-bath for 20 minutes at 44°C (Sakai, 1981), for the same purpose.

IMMUNOENZYMATIC SANDWICH TECHNIQUE TO DETECT ANTI -*VIBRIO ANGUILLARUM* ANTIBODIES IN THE EPIDERMIS

The devices used were the same as described for the light microscopy staining with the PAP method (see earlier in the appendix). As most of the steps of the method were the same of the above cited technique, only the differences introduced will be described.

From step 1 to 4 the procedures were the same :

5) the *Vibrio anguillarum* antigen was aplied as small drops over the sections. For control purposes some sections were only covered with tris saline or with tryptic soy broth; the incubation time was 20 minutes at room temperature;

6) three 10 minutes washings in tris saline;

7) fixation of the antigen in pre-cooled (-2°C) 95% ethanol for 30 minutes;

8) three 5 minutes washings in tris saline;

9) the rabbit serum anti-Vibrio anguillarum was overlaid on the sections at several dilutions (1: 10 to 1: 800) in tris saline with 1% normal swine serum. Normal rabbit serum was applied at the same dilutions, to control the specificity of the reaction. 20 µl of diluted trout mucus were added to 500 µl aliquots of the diluted sera (see 6.1.9);

10) From this step onwards the procedure was the same as for the light microscopy staining with the PAP method (steps 9 to 16). The incubation times, however, were slightly reduced as follows:

- swine serum anti-rabbit IgG - 30 minutes
- PAP complex - 45 minutes
- DAB (0.05% with 0.01% H_2O_2) - 10 minutes.

Morphological And Cytochemical Studies On The Skin Of Rainbow Trout, Salmo Gairdneri.Richardson

Maria da Conceição C.V. Peleteiro

Volume II

A thesis presented for the degree of Doctor of Philosophy in the University of Stirling

1987

FIGURE 1 - Recirculating water system at the Veterinary School (Lisbon) where the fish were kept during immunization experiments.

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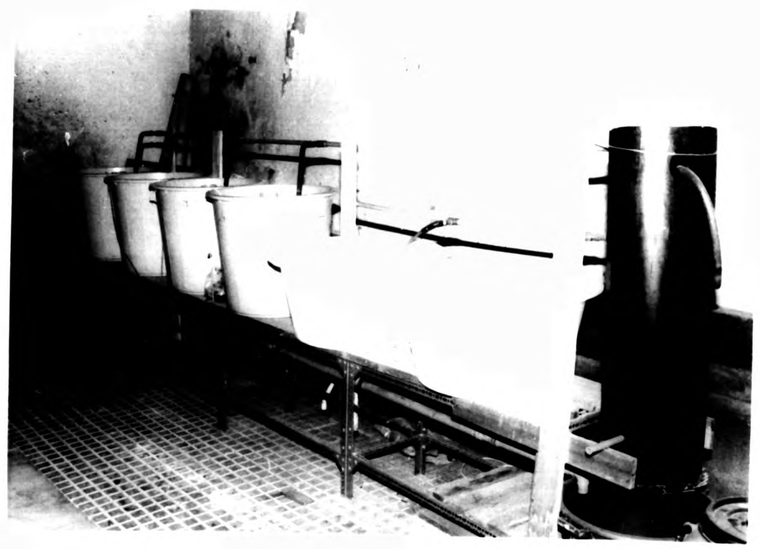
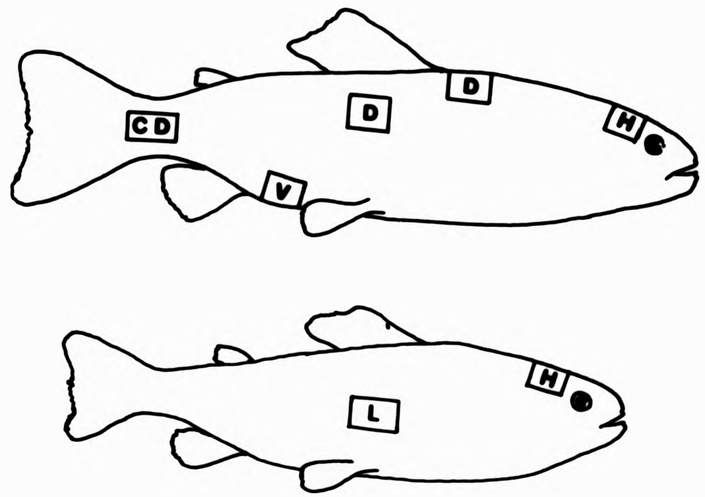


FIGURE 2 - Saprolegnia skin lesions in a female rainbow trout.

FIGURE 3 - Outline drawing of adult and juvenile rainbow trout, showing the areas from which skin samples were taken. H - head skin; D - dorsal skin; V - ventral skin; CD - caudal skin; L - lateral skin.



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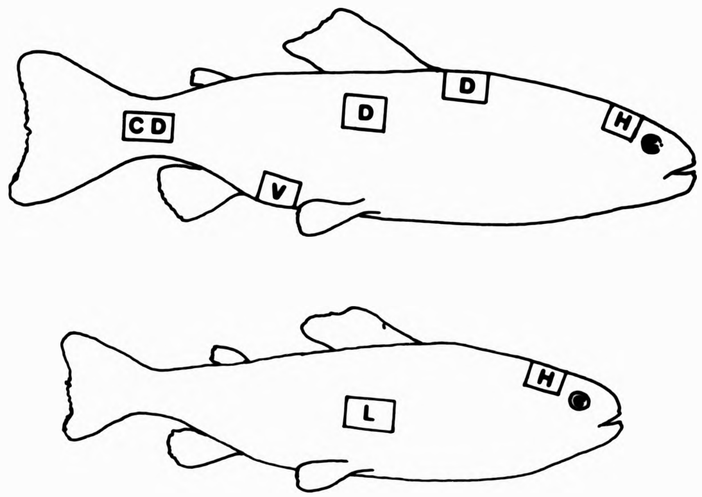


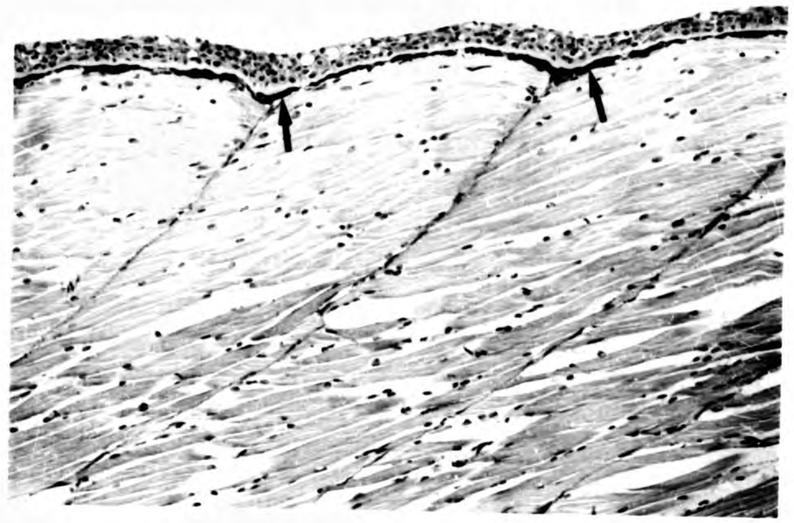
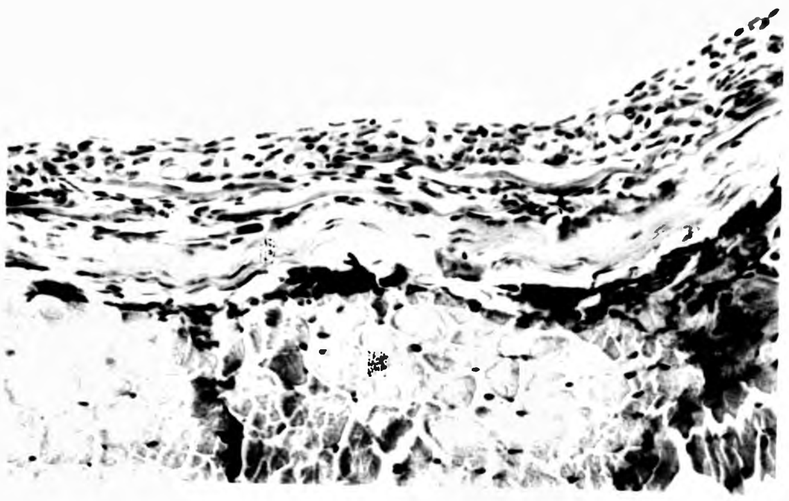


FIGURE 4 - Skin of rainbow trout fry. Note prominent layer of melanin-containing cells just underneath the thick basement membrane (arrows). The epidermis is formed by only a few cell layers with small mucous cells. H.E. x 160.

FIGURE 5 - Skin of very young rainbow trout (±30 weeks). Note the conspicuous dermis (D) and muscle layer (M) and the small scales (arrows) within scale beds. The epidermis is still very thin. H.E. x 250.



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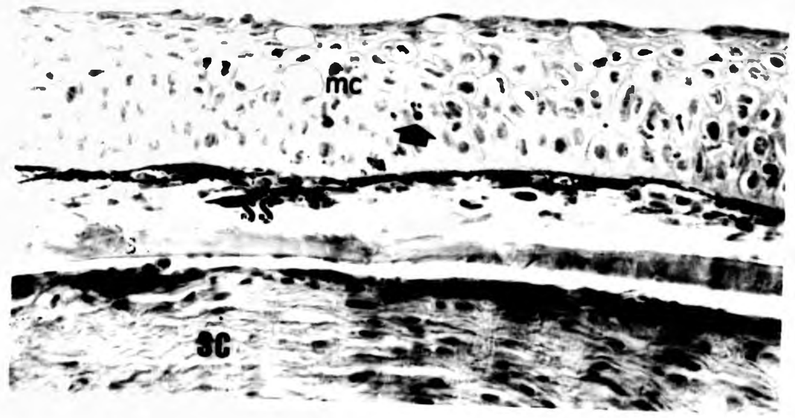


FIGURE 6 - Skin of a juvenile rainbow trout. Note the increase in the size of the scales (s) and the separation between the two dermal strata : stratum spongiosum (SS) in which the scales are found and stratum compactum (SC). The melanin-containing cells still form a continuous layer, but the basement membrane is less obvious than in younger fish. The epidermis is now formed by several layers of epidermal cells and mucous cells (mc). Melanin-granules (arrow) can occasionally be seen scattered in the epidermis. H.E. x 250.

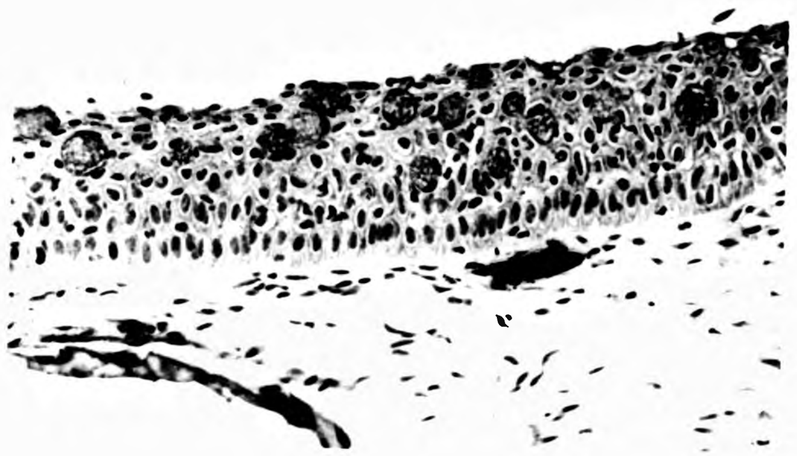
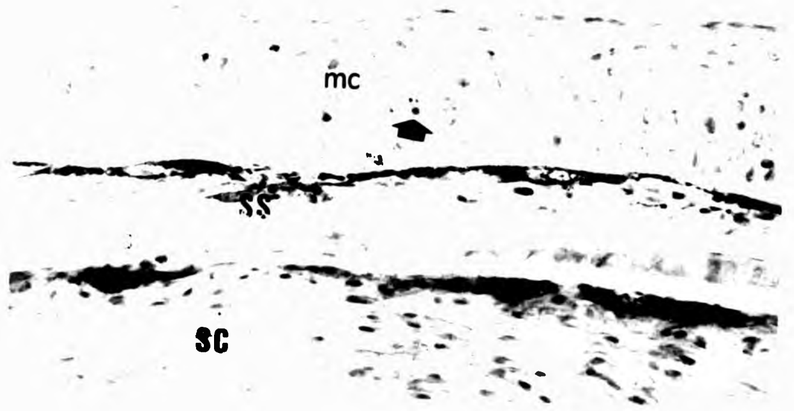


FIGURE 7 - Ventral skin of juvenile rainbow trout. Note the low amount of melanin--containing cells in the dermis. The mucous cell contents are distinctively stained. H.E. x 250.



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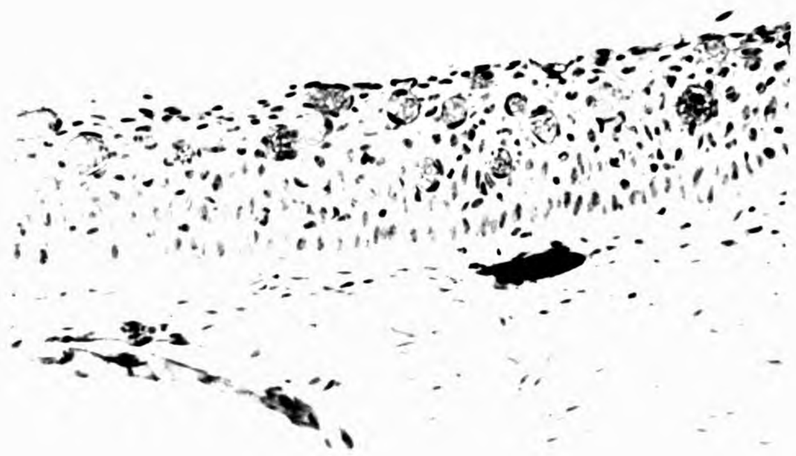

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FIGURE 8 - Head skin of a juvenile rainbow trout which was submitted to a vaccination procedure by direct immersion (D.I.) in a Vibrio anguillarum vaccine. Note the thick epidermis, with sparse mucous cells. A clear infiltration of mononucleated cells, with darkly staining nuclei, can be seen on top of the basement membrane and above the basal layer of columnar epidermal cells (arrows). As the cells reach the surface they become more rounded and at the surface they are clearly flattened. Melanin--containing cells are very numerous in an extremely narrow stratum spongiosum. The stratum compactum is very thick and no scales are seen in the skin in this area. H. E. x 160.

FIGURE 9 - Skin of a sexually mature rainbow trout female. Note the epidermis with a clear basal layer, over which numerous small cells with darkly staining nuclei can be seen. Some of these cells appear to be located along the basement membrane (arrows). The content of the numerous mucous cells is totally unstained. H.E. x 160.

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FIGURE 10 - Skin of a juvenile rainbow trout, which was vaccinated by D.I. Note the infiltration with small cells with darkly staining nuclei in the basal epidermal layers. The mucous cell content is moderately stained. H.E. x 250.

FIGURE 11 - Dorsal skin of sexually mature rainbow trout male. Note the pronounced foldings of the basement membrane due to the marked dermal papillae. The number of mucous cells in the thickened epidermis is greatly decreased. Note the position of the scales (s) deeply buried in a thickened dermis. H.E. x 63.

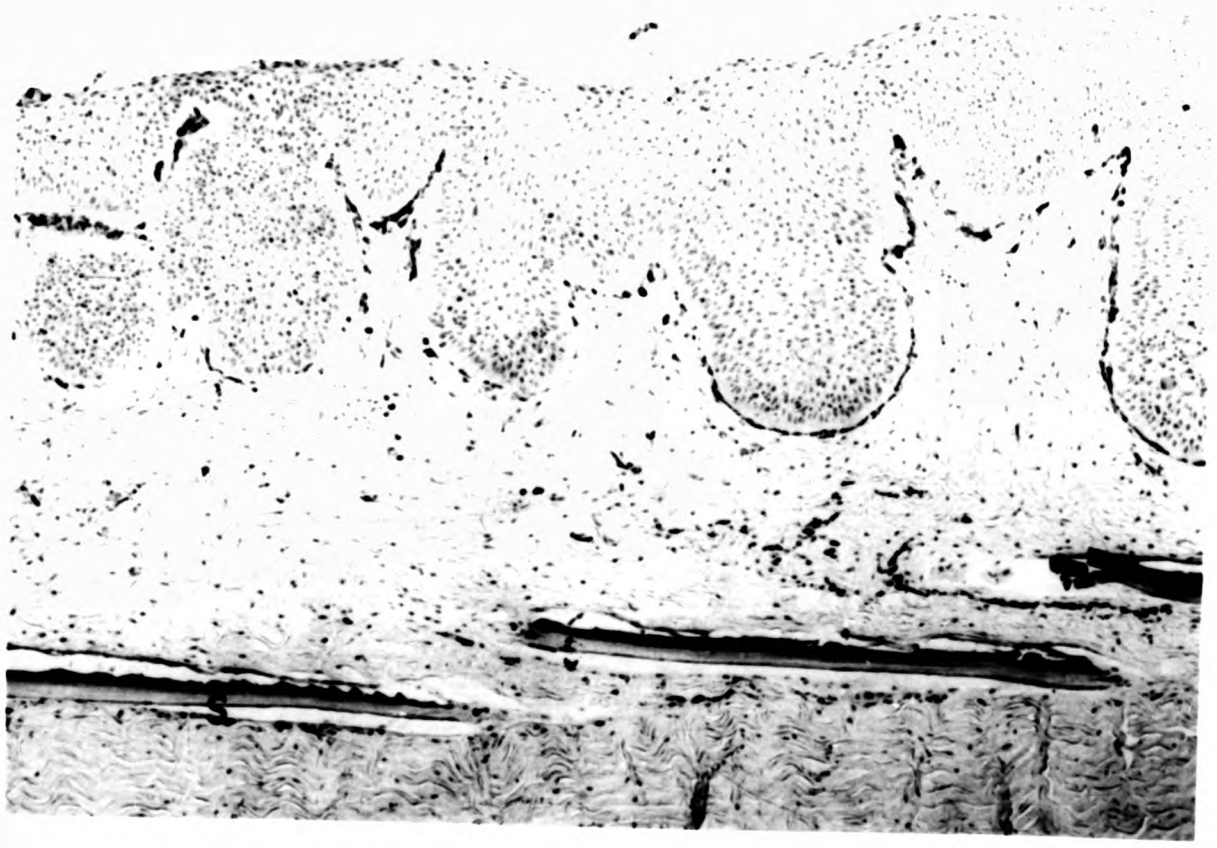

FIGURE 12 - Ventral skin of a sexually mature rainbow trout male. Note the large number of unstained mucous cells in the thickened epidermis. Numerous small cells with darkly stained nuclei can be seen in the deeper layers of the epidermis (arrows). The melanin-containing cells in the dermis are rare, as expected in the ventral skin. H.E. x 160.

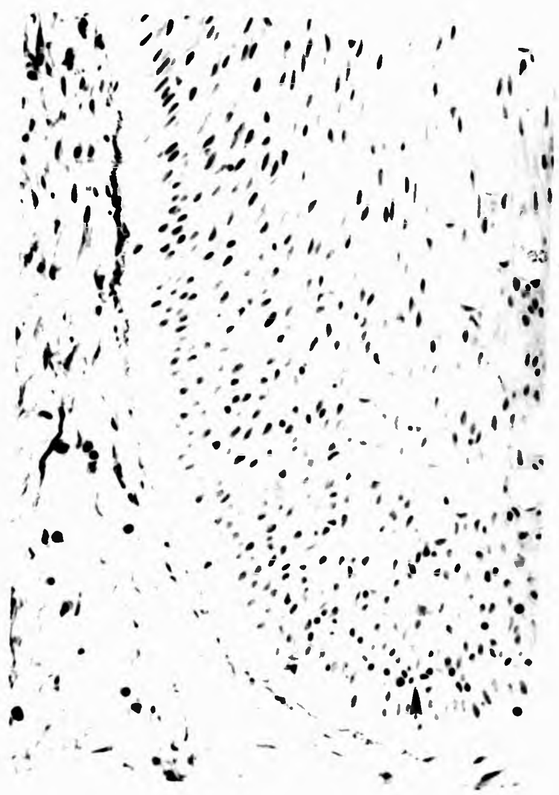

FIGURE 13 - Skin of a sexually mature rainbow trout male. Note the areas where small groups of infiltrating cells can be seen (arrowheads) separating the epidermal layers. H.E. x 250.

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FIGURE 13 - Skin of a sexually mature rainbow trout male. Note the areas where small groups of infiltrating cells can be seen (arrowheads) separating the epidermal layers. H.E. x250

FIGURE 14 - Skin of a sexually mature rainbow trout female. Note the superficial epidermal cells, which appear round and clear, not assuming the typical flattened profile. H.E. x 250.

FIGURE 15 - Skin of a sexually mature rainbow trout female. Note the intercellular spaces between epidermal cells (spongiosis) and the infiltration of small cells with darkly staining nuclei throughout the epidermis. H.E. x 160.

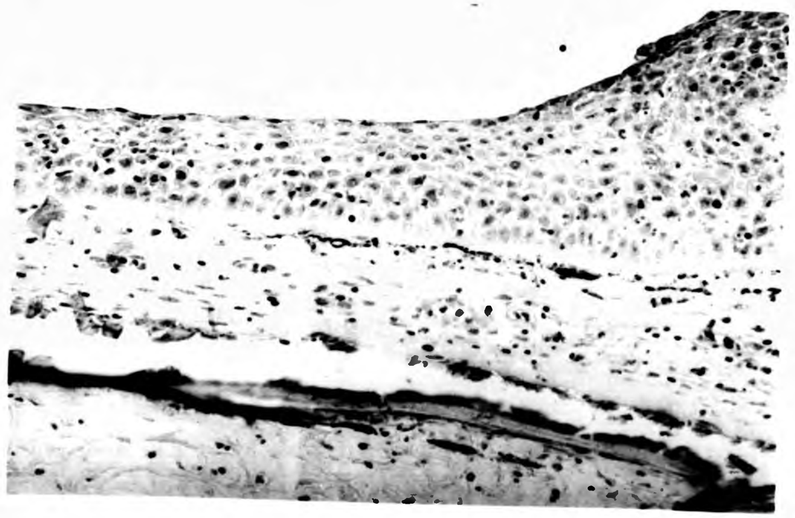

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FIGURE 16 - Skin of a sexually mature rainbow trout female. Note the extensive spongiosis in the epidermis and the marked cellular infiltration at that level.The dermal stratum spongiosum also shows considerable infiltration with cells which apparently are of the same type as those found in the epidermis. H. E. x 160.

FIGURE 17 - Magnification of figure 16. Note the large granules which are clearly seen inside most epidermal cells. No increased sloughing is apparent, as the superficial cells are typically flattened. H.E. x 400.

FIGURE 16 - Skin of a sexually mature rainbow trout female. Note the extensive spongiosis in the epidermis and the marked cellular infiltration at that level.The dermal stratum spongiosum also shows considerable infiltration with cells which apparently are of the same type as those found in the epidermis. H.E. x 160.

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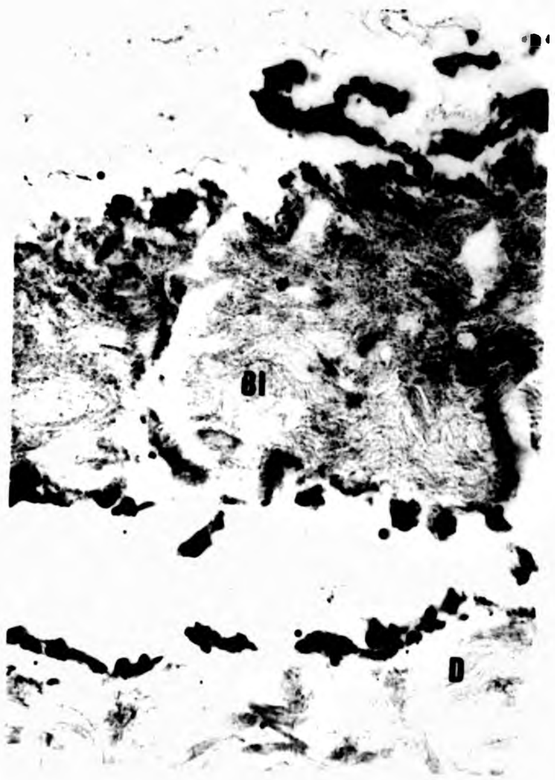

FIGURE 18 - Skin of a sexually mature rainbow trout female shown in figure 2, suffering from a severe Saprolegnia infection. Note the absence of epidermis as well as basement membrane. Numerous hyphae were located at the skin surface (unseen in the photograph). Bacterial infiltrates (BI) can be seen above the dermis (D). H.E. x 250.

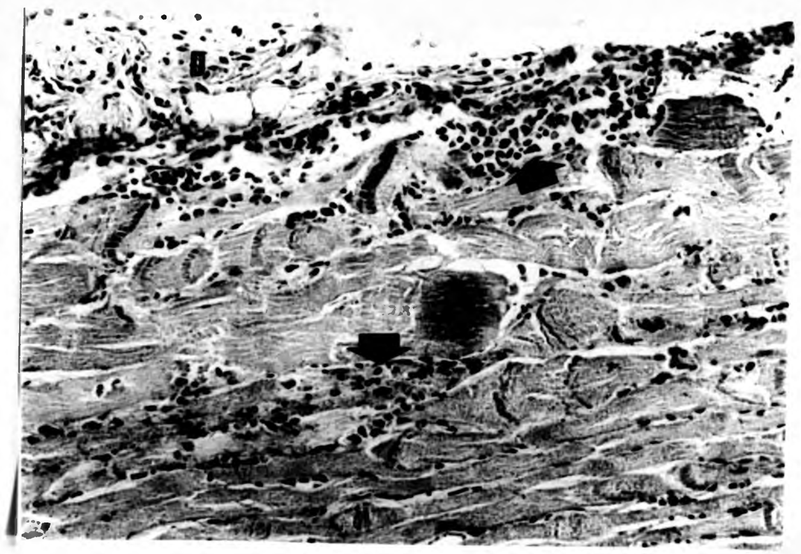

FIGURE 19 - Muscle fibres located underneath severe Saprolegnia skin lesions of the sexually mature rainbow trout female shown in figure 2. Note the irregular diameter showed by some fibres and the cellular infiltration, representing an inflammatory reaction. Some fibres seem to be undergoing a digestive process by the inflammatory cells (arrows). Dermis (D). H.E. x 160.

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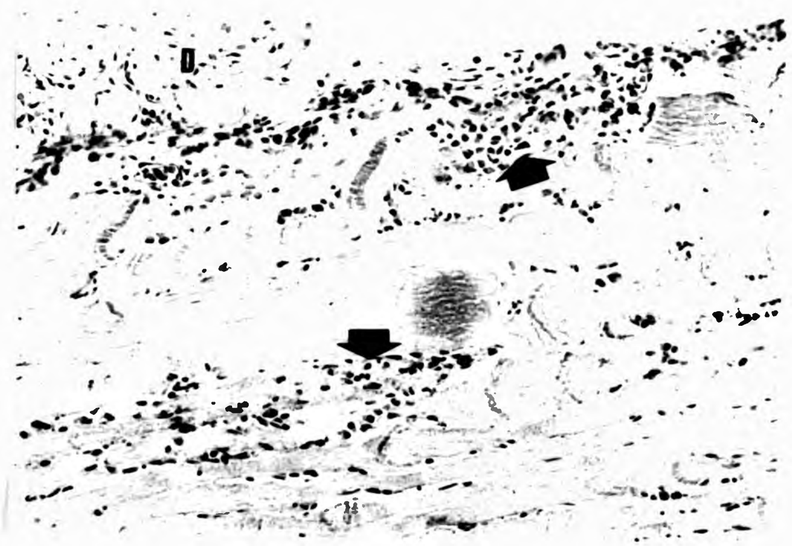

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FIGURE 20. Branchial arch of a juvenile fish, which was vaccinated by D.I.. Note how the branchial arch epithelium is similar to the skin epidermis, containing mucous cells and highly infiltrated with small cells with darkly staining nuclei. The supporting connective tissue shows some eosinophilic granular cells (arrows) frequently found in this area. H.E. x 250.

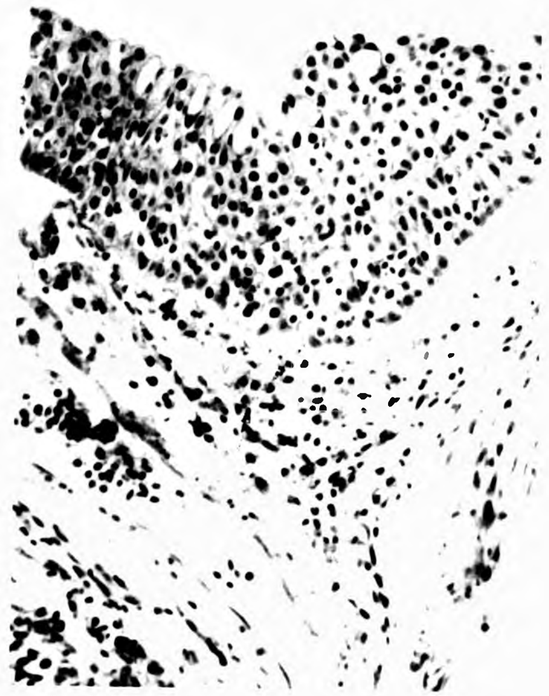

FIGURE 21 - Branchial arch of a juvenile fish, which was used as control in the vaccination experiment. The epithelium is infiltrated with small cells with darkly staining nuclei. On the left a taste bud can be seen. H.E. x 400.

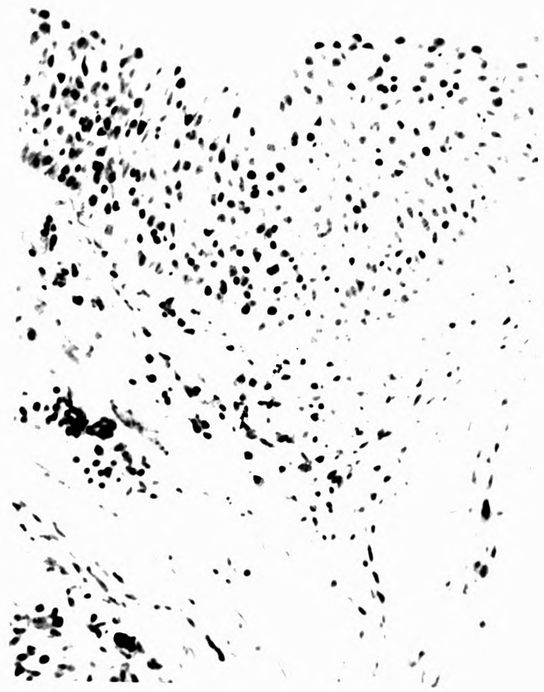

FIGURE 20. Branchial arch of a juvenile fish, which was vaccinated by D.I.. Note how the branchial arch epithelium is similar to the skin epidermis, containing mucous cells and highly infiltrated with small cells with darkly staining nuclei. The supporting connective tissue shows some eosinophilic granular cells (arrows) frequently found in this area. H.E. x 250.

FIGURE 21 - Branchial arch of a juvenile fish, which was used as control in the vaccination experiment. The epithelium is infiltrated with small cells with darkly staining nuclei. On the left a taste bud can be seen. H.E. x 400.

FIGURE 22 - Base of a primary lamella of the gill of a sexually mature female. Note the numerous mucous cells in the superficial epithelium (below) and the extensive infiltration of small cells with darkly stained nuclei up to the base of the secondary lamellae (SL). H.E. 250.

FIGURE 23 - Tip of a primary lamella of the gill of a juvenile fish, used as control in the vaccination experiment. Note the infiltration of small cells with darkly stained nuclei at the extremity of the lamella. H.E. x 160.

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FIGURE 23 - Tip of a primary lamella of the gill of a juvenile fish, used as control in the vaccination experiment. Note the infiltration of small cells with darkly stained nuclei at the extremity of the lamella. H.E. x 160.

FIGURE 24 - Thymus of a rainbow trout fry. Note the lymphoid tissue separated from the gill chamber (GC) by a single layer of epithelial cells, including some mucous cells. The thymocytes are separated from the underlying muscle (M) by a very thin layer of connective tissue. Occasionally, cells with large clear nucleus can be noticed in between the thymocytes (arrowheads). The density of thymocytes seems to be higher in the external area of the organ. H.E. x 160.

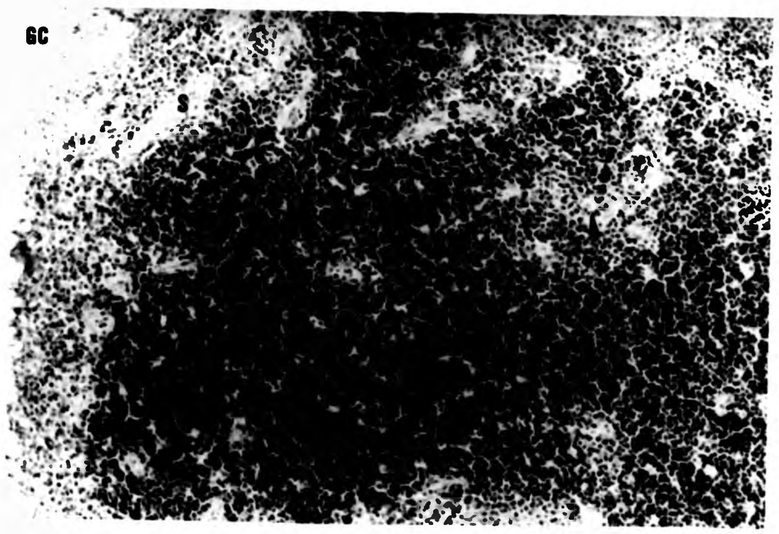

FIGURE 25 - Thymus of a juvenile rainbow trout. Note the large number of mucous cells in the epithelium which separates the thymus from the gill chamber (GC). Small strands of connective tissue are occasionally found (S). A few cells with large clear nucleus (arrowheads) can be seen between the thymocytes. H.E. x 160.

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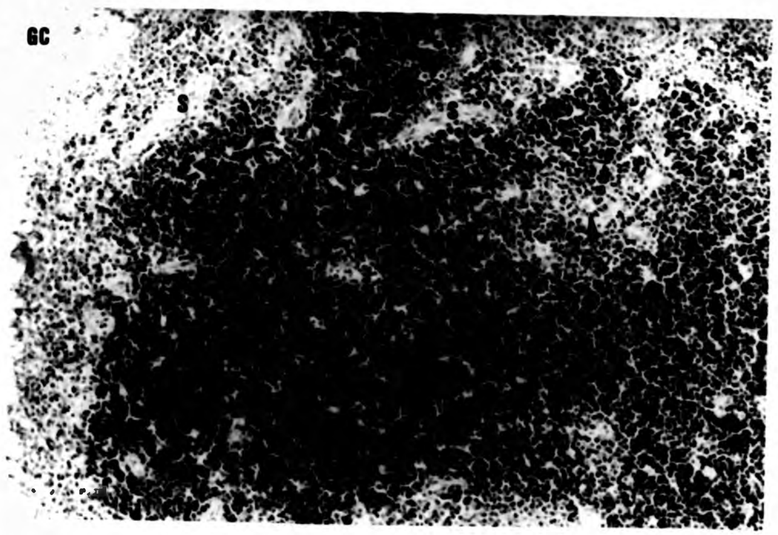

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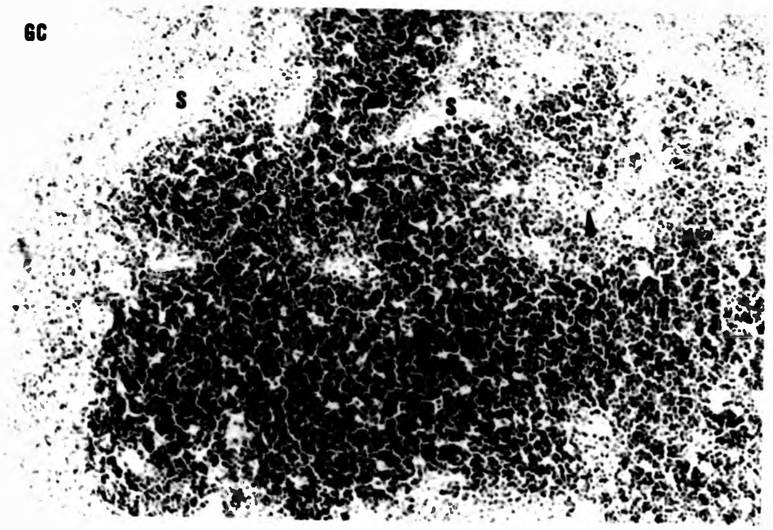

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FIGURE 26 - Electron micrograph of mature female skin. Note the small indentations of the basement membrane which is formed by a continuous dense layer (arrow) separated from the epidermal cells by a lighter zone. Above the basement membrane four mononucleated cells can be seen. Nuclear density suggests that the cells on the left are lymphocytes and the smaller on the right is a section of a macrophage-like cell. Nucleus (n); mitochondria (m); Golgi apparatus (g); lysosome granule (l); filament--containing cell (FCC); dermis (D). x 8 200.

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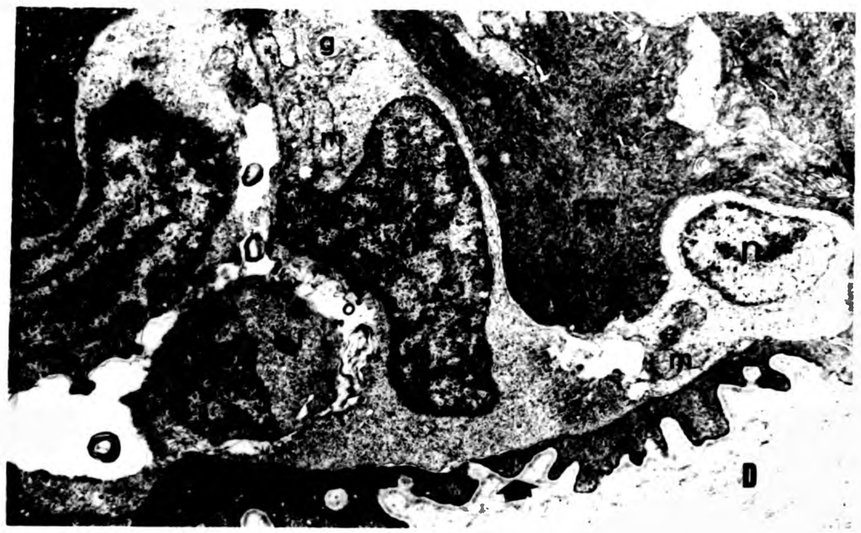

FIGURE 27 - Electron micrograph of mature male skin. Note the complex system formed by the basement membrane. Desmosomes (d) between contiguous filament-containing cells ; dermis (D). x 9600.

FIGURE 28 - Electron micrograph of mature female skin. Note the thin fibrillar elements that can be seen crossing from the lamina densa towards the basal filament--containing cells (FCC). A network of thin reticular fibres can be seen just underneath the lamina densa. Above the basement membrane is a macrophage with a deep clefted moderatly dense nucleus (n), mitochondria (m), Golgi saccules (g) and small electron dense granules with a surrounding clear halo, dispersed in the cytoplasm (arrowheads). Dermis (D). x 8 200.

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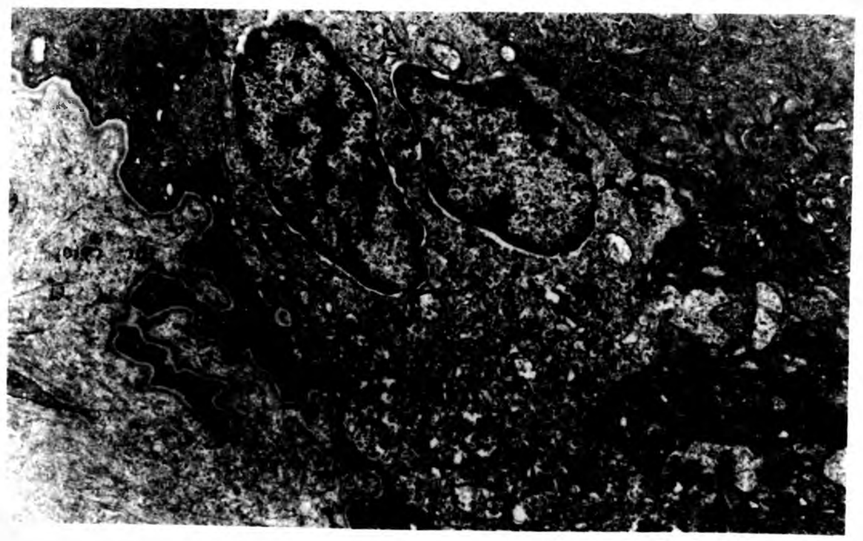


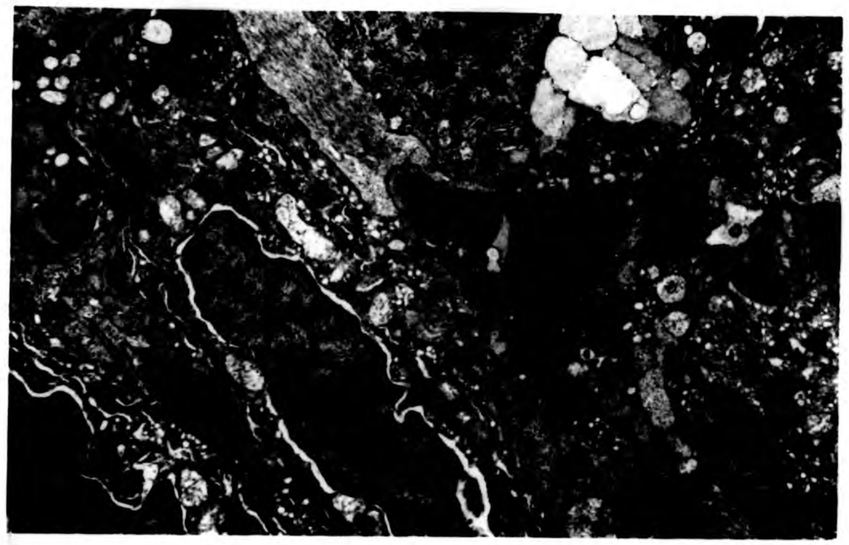
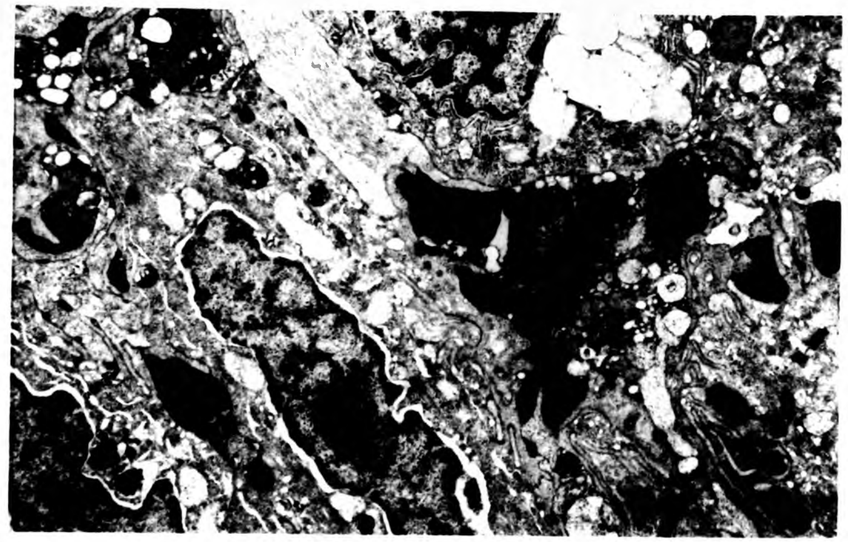


FIGURE 29 - Electron micrograph of a juvenile epidermis. Basal filament-containing cells showing an elongated nucleus (n) with an obvious nucleolus (nc). Note the interdigitations between neighbouring cells (arrows). The electron dense fragments seen in the intercellular space probably represent remains of chromatin from necrotic cells. At the top of the photograph a mucous cell can be seen (M). This juvenile fish was vaccinated by direct immersion in a Vibrio anguillarum vaccine. x 7 600.

FIGURE 30 - Electron micrograph of mature female epidermis. Filament-containing cells with marked interdigitations and desmosomes. The cells show mitochondria (m) and free ribosomes, mainly located in the perinuclear area. Note the bundles of tonofilaments mainly located peripherally in the cytoplasm. Nucleus (n). x 9 600.



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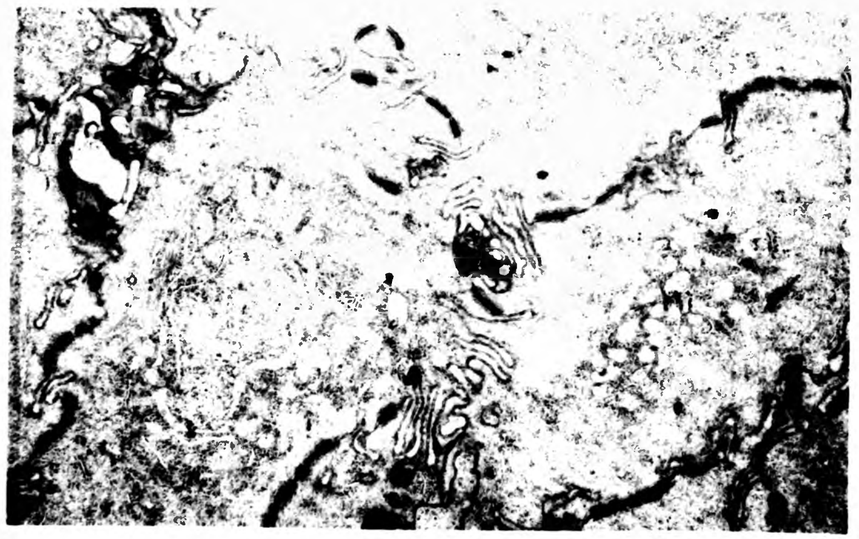

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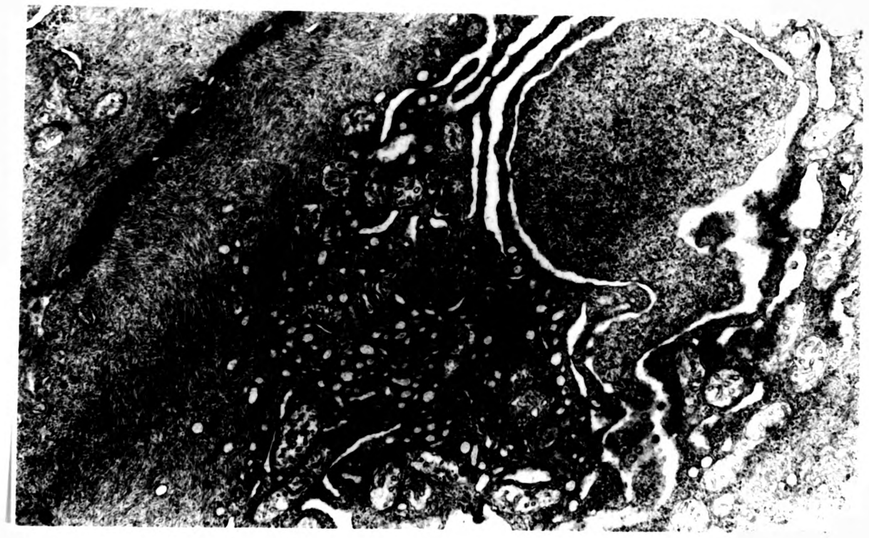
FIGURE 31 - Electron micrograph of mature female skin. Note the internal organization of a filament-containing cell: desmosomes (d); mitochondria (m); smooth surface vesicles (arrowheads); free ribosomes and tonofilaments (t). Nucleus (n). x 15 200.

FIGURE 32 - Electron micrograph of mature female epidermis. Superficial filament-containing cells. Four layers of cells can be seen, numbered from 1 to 4. Note the flattened shape of the cells and the superficial microridges (arrowheads). The FCC's of the third layer show electron dense bodies (b) of heterogenous content. x 7 600.



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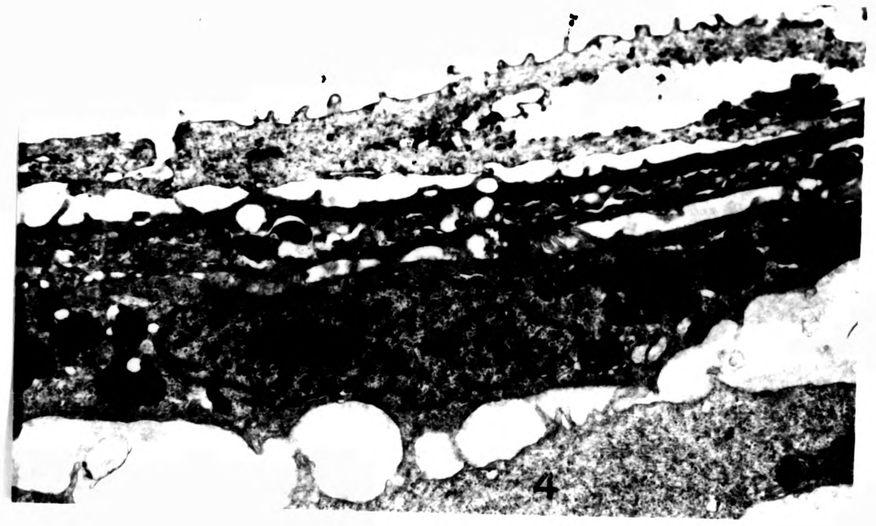

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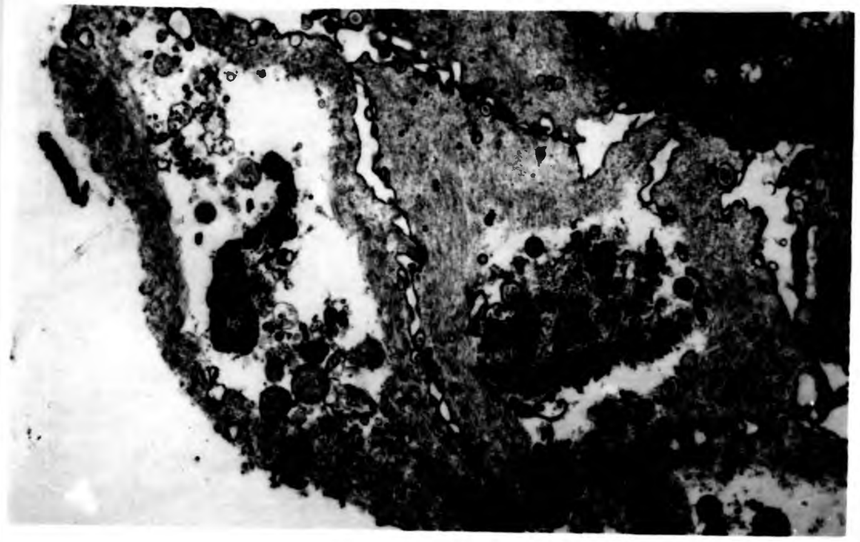
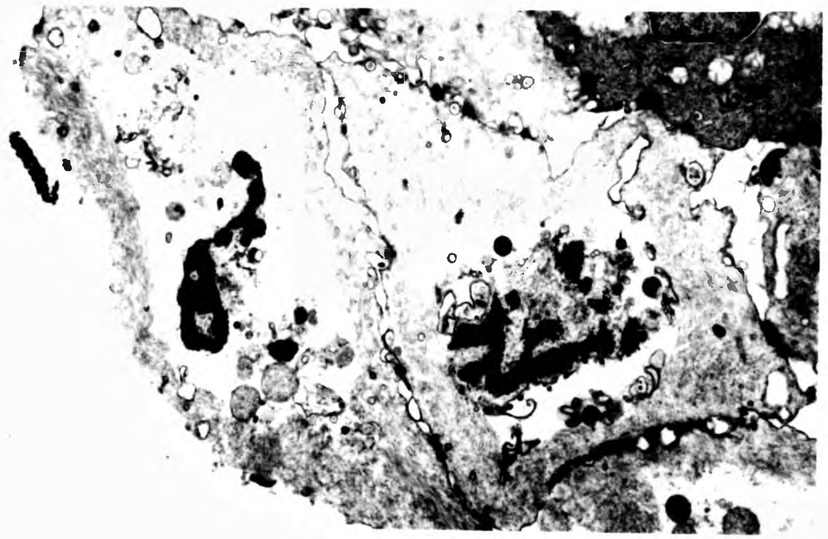


FIGURE 33 - Electron micrograph of mature female epidermis. The filament-containing cells assume a rounded configuration, with loss of cytoplasmic content, especially in the perinuclear area. The nuclear chromatin is partly condensed and there is loss of the nuclear membrane. The skin surface is to the left and the FCC which is seen in the upper right angle is normal. x 7 600.

FIGURE 34 - Electron micrograph of a section of mature male skin. Note the folded basement membrane. Between the basal filament-containing cells (FCC), reduced intercellular spaces (is) are formed, and the interdigitating folds of their cell membranes appear in contrast to the low electron dense background. x 8 800.



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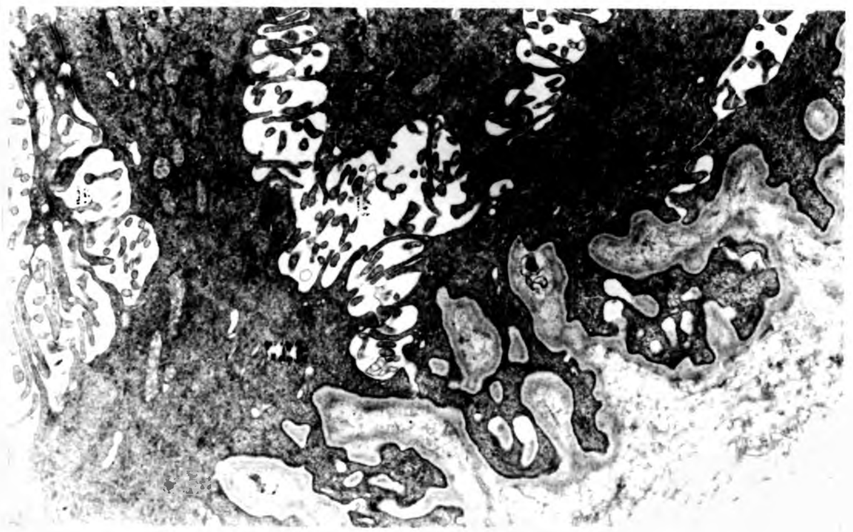

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FIGURE 35 - Electron micrograph of mature male epidermis. Note the wide intercellular spaces (is) and the filament-containing cells with a rounded outline, connected by desmosomes (arrows). In the lower left a macrophage can be seen (MA), with numerous dense bodies. Nucleus (n), nucleolus (nc). x 7 900.

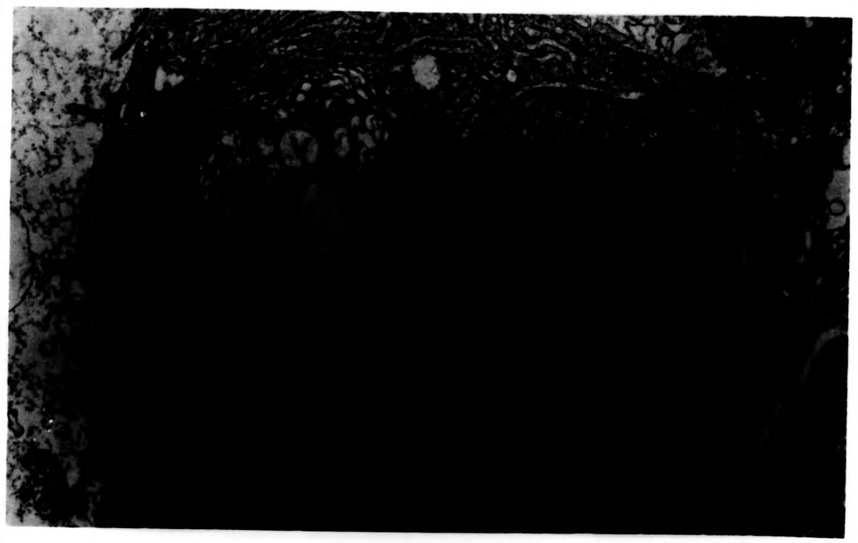

FIGURE 36 - Electron micrograph of mature female epidermis showing an immature mucous cell. Note the abundant rough endoplasmic reticulum and a few vesicles (v) with flocculent material. Numerous saccules of the Golgi apparatus can be seen around the vesicles. Nucleus (n). Mitochondria (m). x 10 000.

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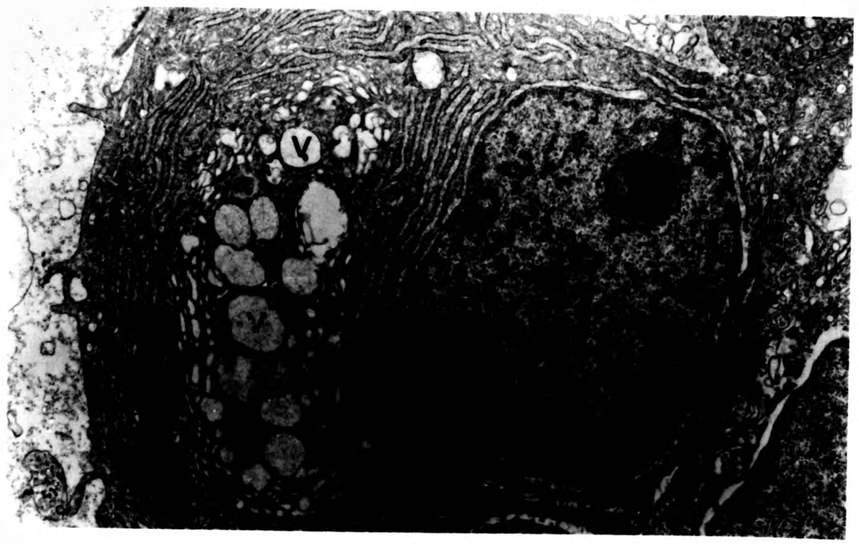

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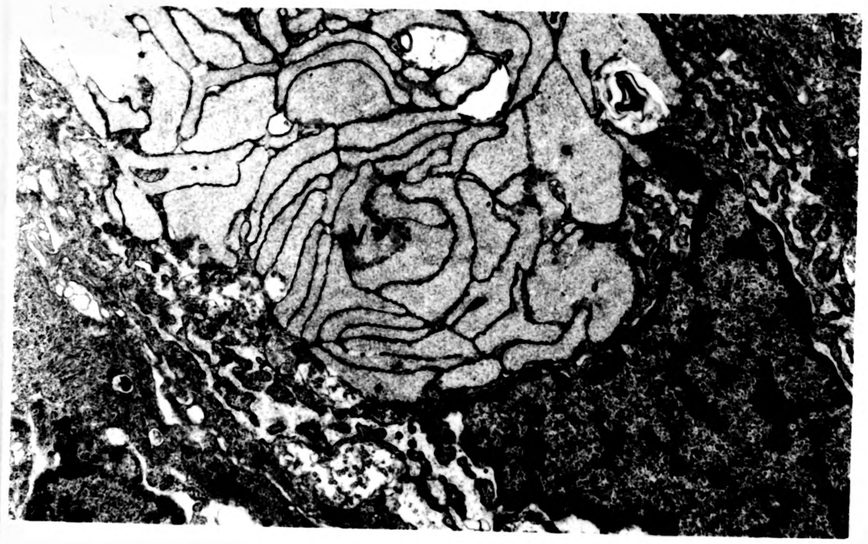


FIGURE 37 - Electron micrograph of mature female epidermis showing maturing mucous cell, with large mucous vesicles (v) which compress the nucleus (n). x 11 400.

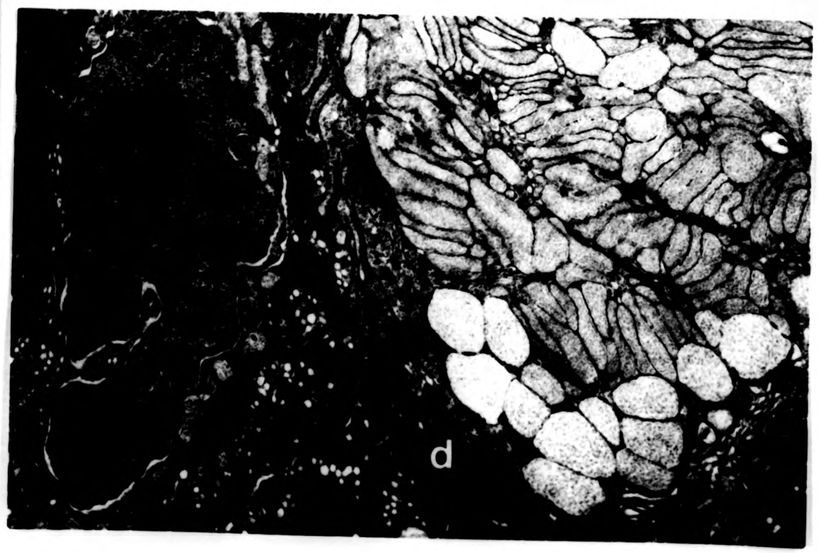
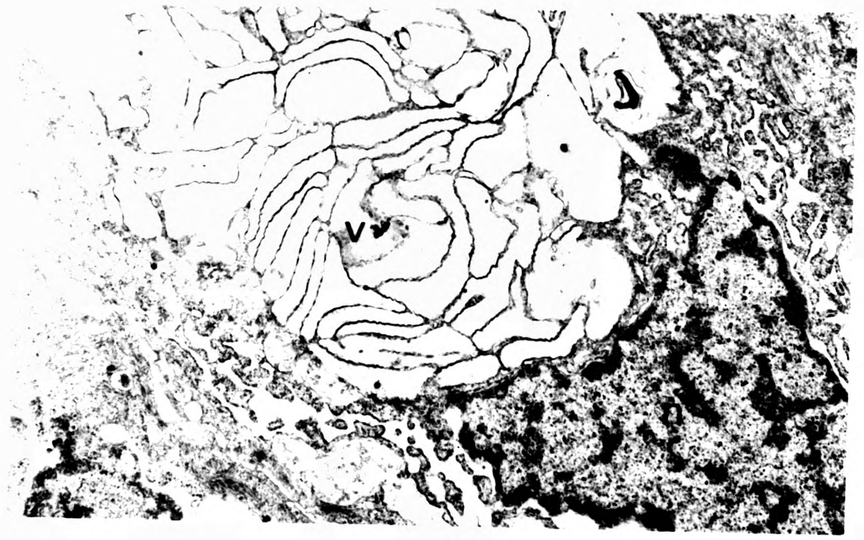


FIGURE 38 - Electron micrograph of mature female epidermis showing a mature mucous cell (right) and a filament-containing cell (left). Note the different sizes of the mucous vesicles and their variable electron density. Desmosomes (d) can be seen between the mucous cell and apposed filament-containing cells. x 10 000.



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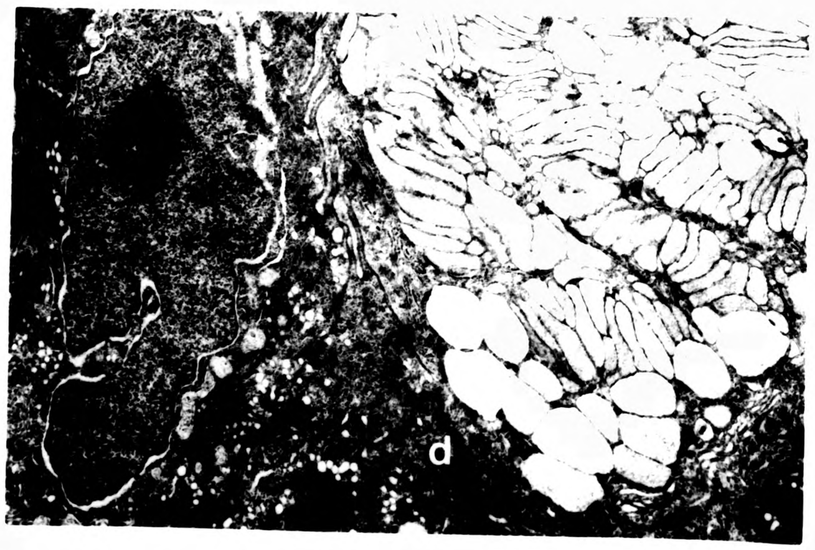


FIGURE 39 - Electron micrograph of juvenile epidermis showing a mucous cell releasing its content onto the surface. The vesicles appear fused together leaving only a few independent profiles. The mucous cell is emerging between filament-containing cells (FCC). x 9600.

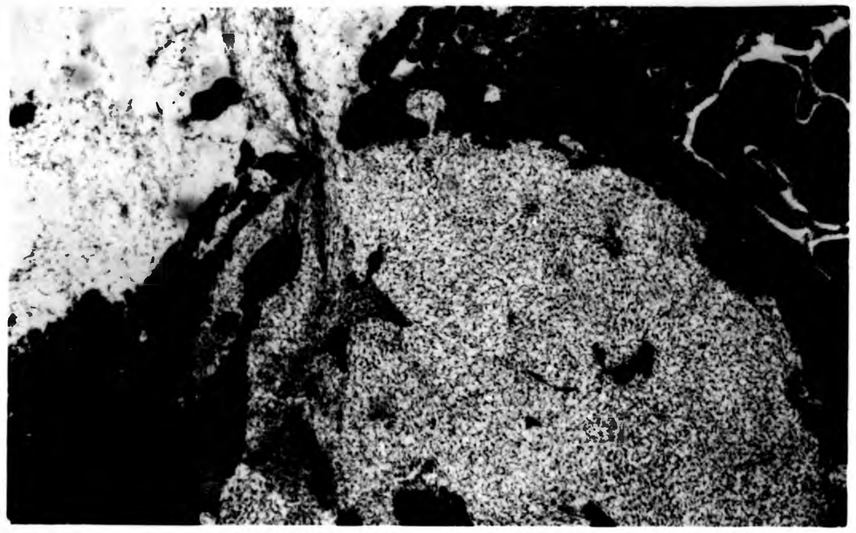
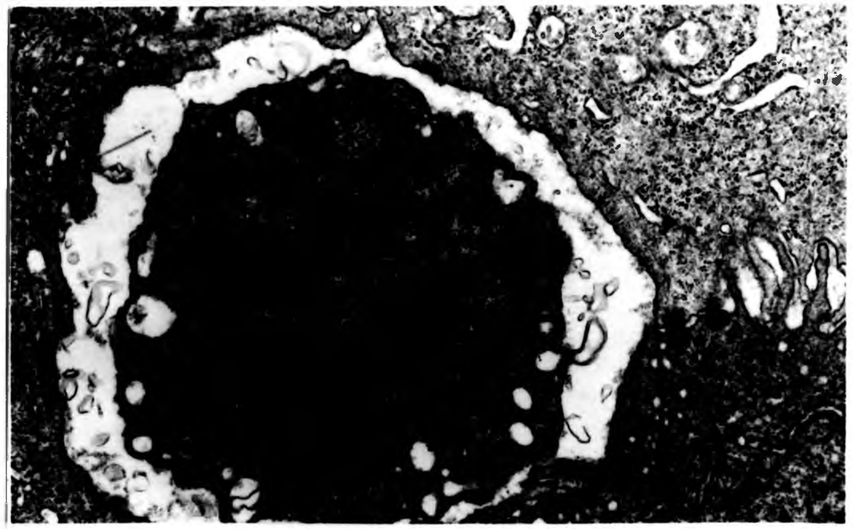
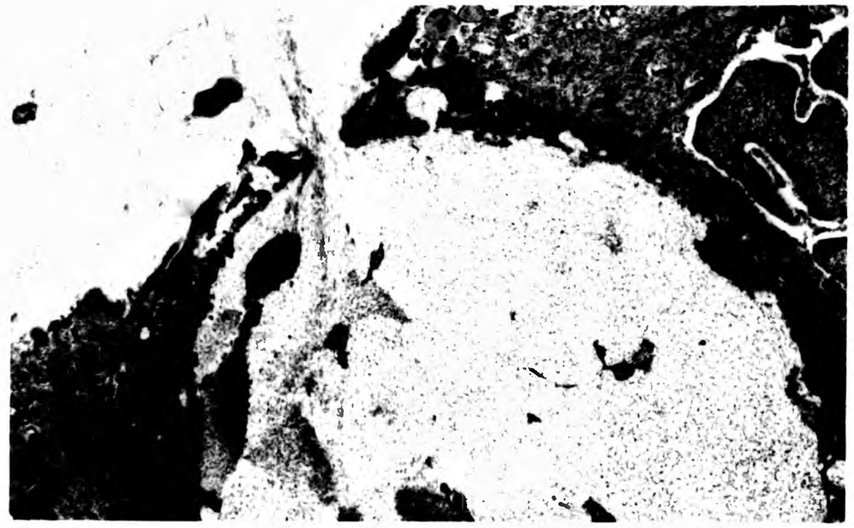


FIGURE 40 - Electron micrograph of mature female epidermis. A lymphocyte-like cell is present with moderately dense nucleus (n), with deep clefts that account for the two portions of nuclear material. A reduced rough endoplasmic reticulum, few mitochondria (m) and frequent rough coated vesicles (v) can be seen in the cytoplasm. Note the surrounding intercellular space. x 11000.





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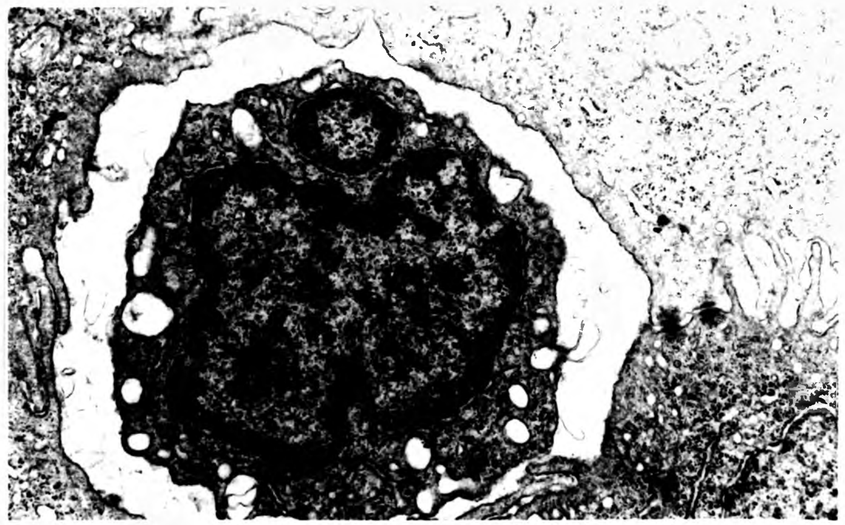

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m

d

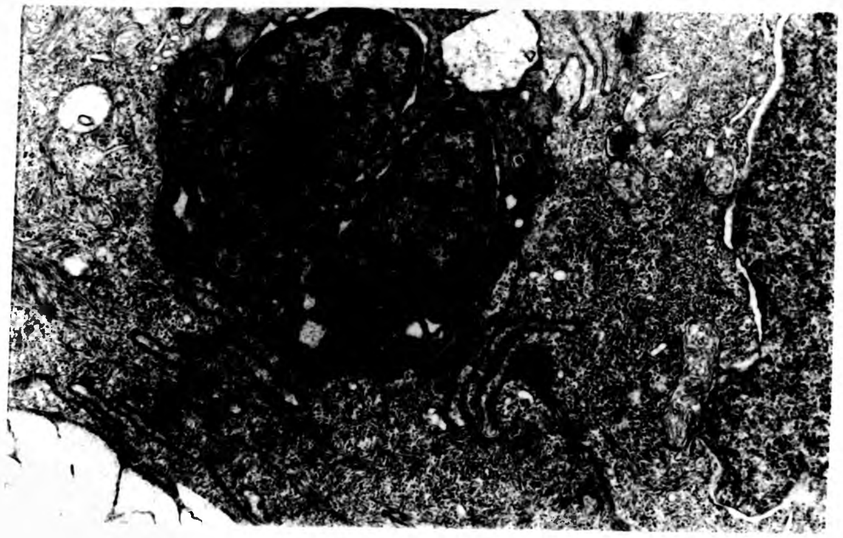
FIGURE 41 - Electron micrograph of mature female epidermis, showing a lymphocyte-like cell with a dense nucleus (n) and conspicuous nucleolus. Mitochondria (m), rough endoplasmic reticulum and moderately dense bodies (b) can be seen in the cytoplasm. Note the intimate relationship with the neighbouring filament-containing cells with no intercellular space. x 17 100.

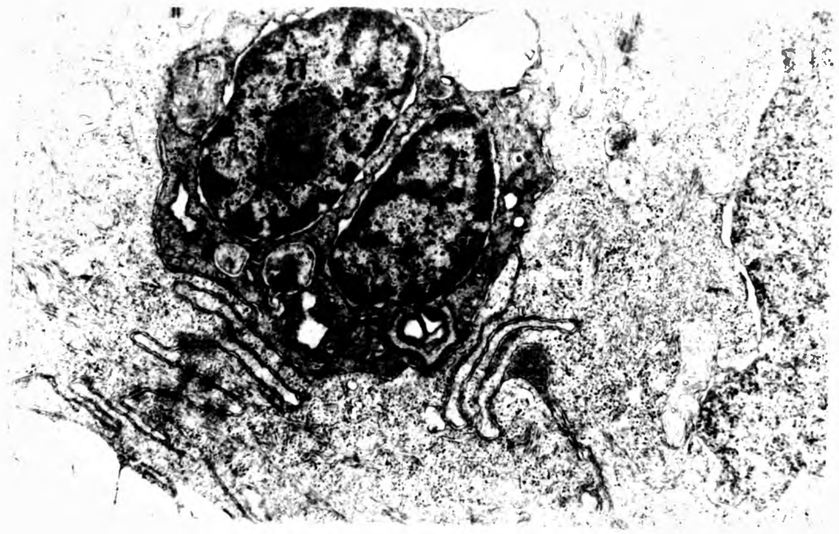
FIGURE 42 - Electron micrograph of a section of mature female epidermis, showing a lymphocyte-like cell,with dense nucleus (n) and nucleolus. In the cytoplasm, small lysosome-like bodies (arrows) and large dense bodies (b) can be seen. The cell outline shows a few pseudopodia (ps). x 15 600.

n

d

ps





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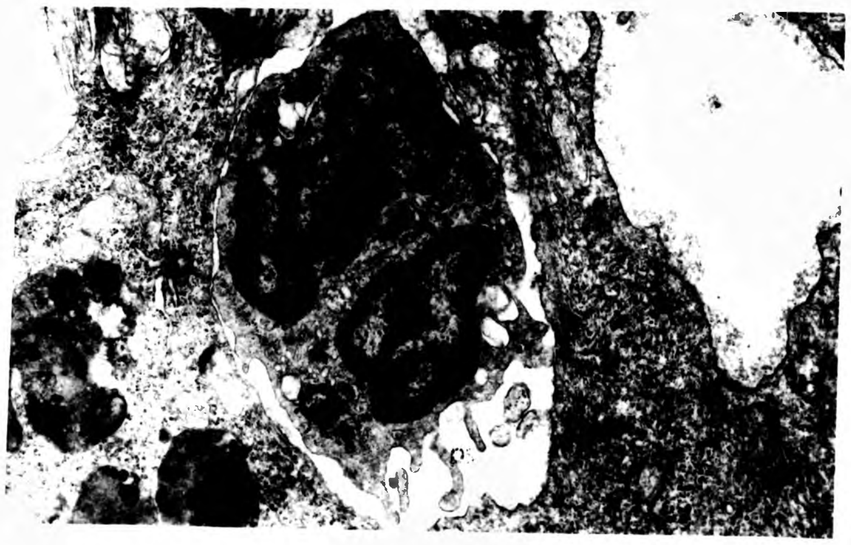

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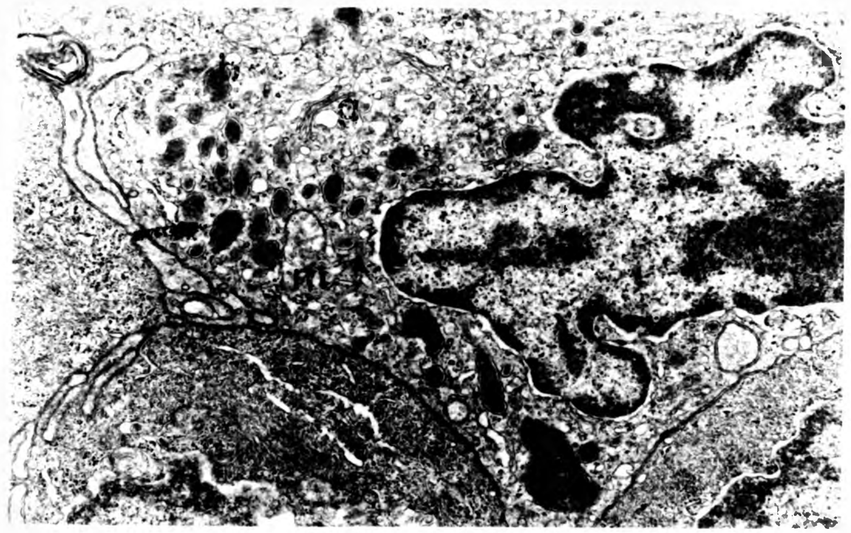
FIGURE 43 - Electron micrograph of mature female epidermis, showing a macrophage-like cell, with a large nucleus (n). The cytoplasm is filled with moderately dense bodies (b), presumably lysosomes, and small vesicles. A phagocytosed melanin granule can be seen inside a phagosome (arrow). Mitochondria (m) and Golgi apparatus (g) can also be seen. x 13 000.

FIGURE 44 -Electron micrograph of mature male epidermis showing a macrophage-like cell, with a very deeply clefted nucleus (n) and elongated mitochondria (m). Small vesicles, rough endoplasmic reticulum and free ribosomes can also be seen in the cytoplasm. x 16 000.



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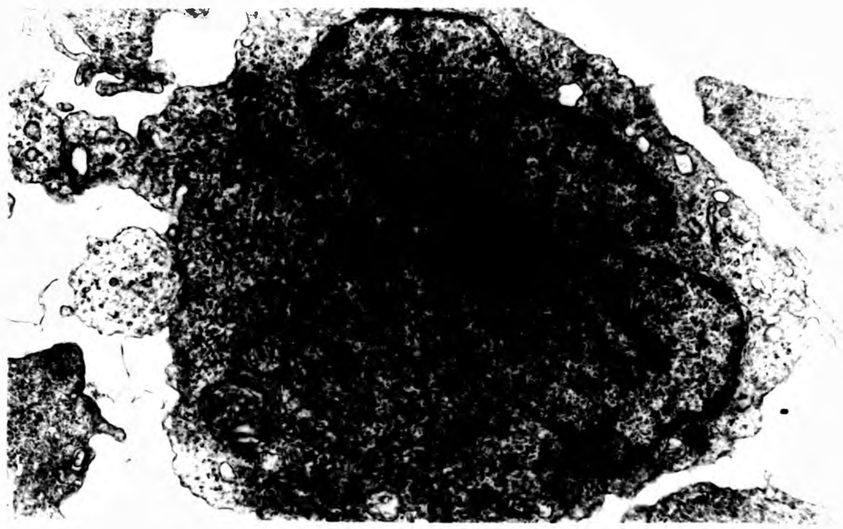

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FIGURE 45 - Electron micrograph of mature female skin showing a cell crossing the basement membrane (arrows). The nucleus (n) is moderately dense and the cytoplasm shows mitochondria (m), lysosome-like bodies (l), filaments (f), small vesicles and Golgi saccules (g). Dermis (D). x 36 000.

FIGURE 46 - Electron micrograph of juvenile skin, showing the basement membrane and a lymphocyte-like cell on the epidermal side (E). In the dermal side numerous reticular fibres can be seen anchoring into the basal lamina. Dermis (D). (*) folding of the basement membrane . This juvenile was vaccinated by direct immersion in a Vibrio anguillarum vaccine. x 18 000.

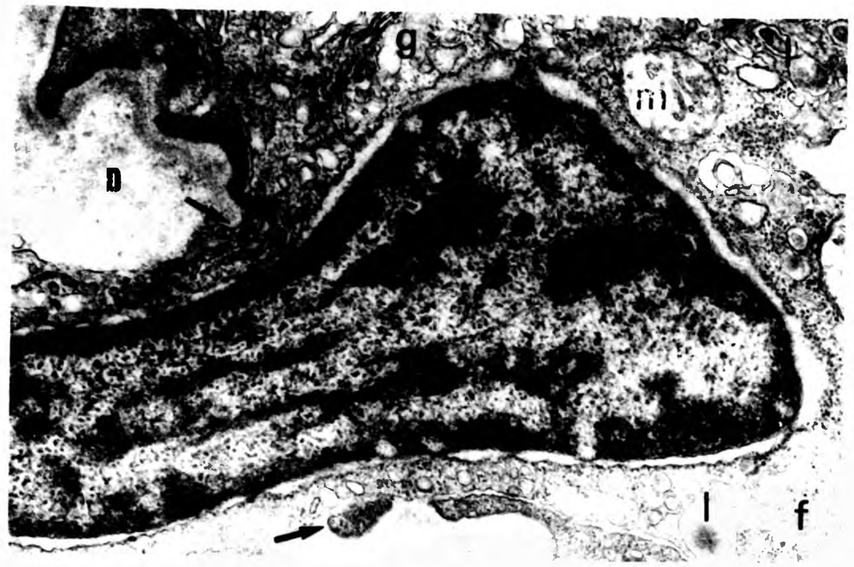

FIGURE 45 - Electron micrograph of mature female skin showing a cell crossing the basement membrane (arrows). The nucleus (n) is moderately dense and the cytoplasm shows mitochondria (m), lysosome-like bodies (l), filaments (f), small vesicles and Golgi saccules (g). Dermis (D). x 36 000.

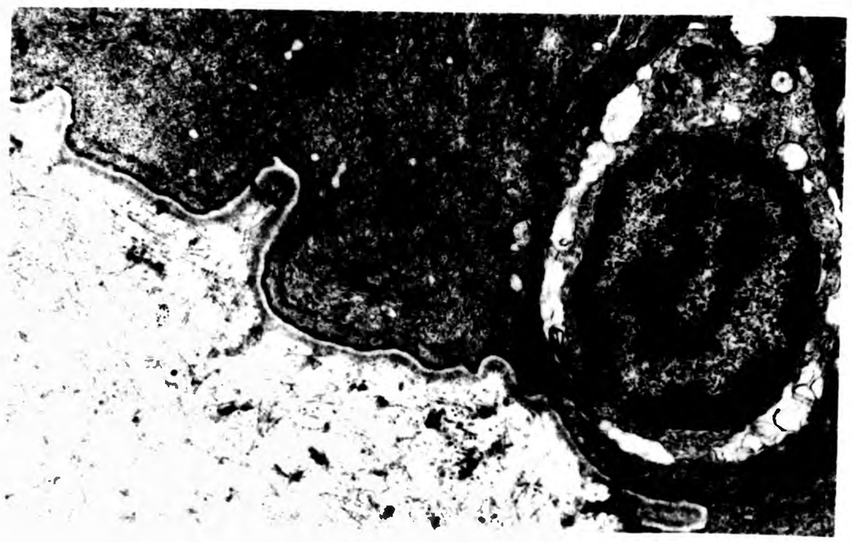

FIGURE 46 - Electron micrograph of juvenile skin, showing the basement membrane and a lymphocyte-like cell on the epidermal side (E). In the dermal side numerous reticular fibres can be seen anchoring into the basal lamina. Dermis (D). (*) folding of the basement membrane . This juvenile was vaccinated by direct immersion in a Vibrio anguillarum vaccine. x 18 000.

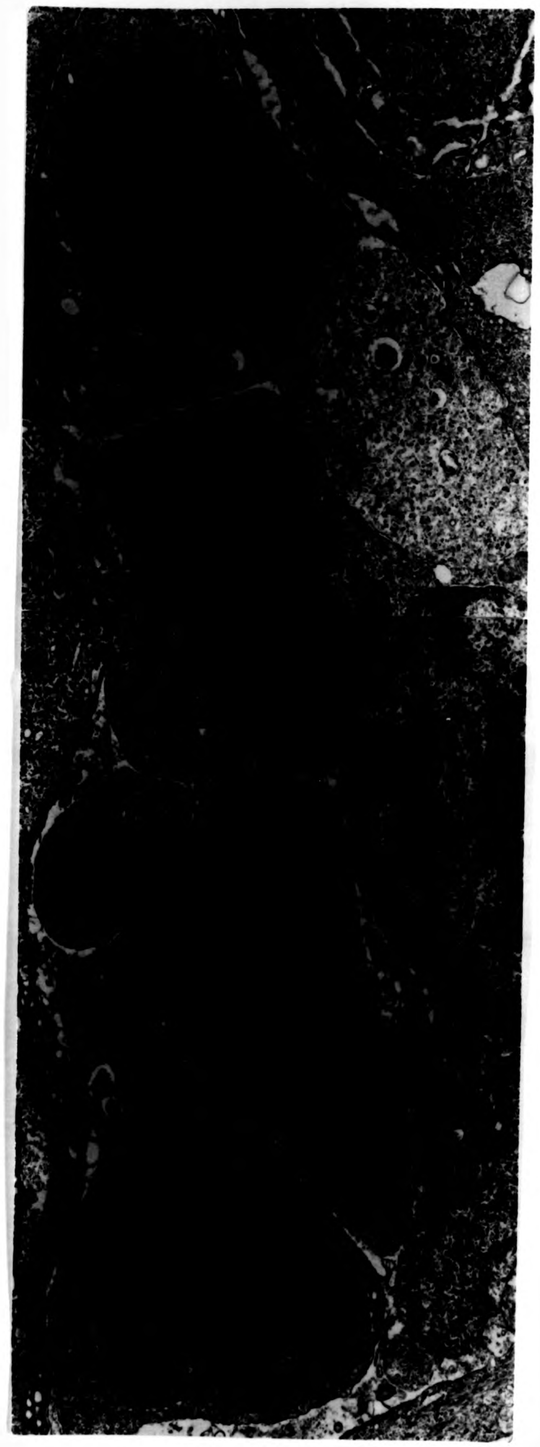

FIGURE 47 - Electron micrograph of mature female epidermis showing an aggregate of lymphocyte-like cells. All cells have a high nuclear density and reduced cytoplasm. Their internal structure shows mitochondria, very few lysosome-like bodies (arrow), small vesicles and numerous free ribosomes. x 9 800.

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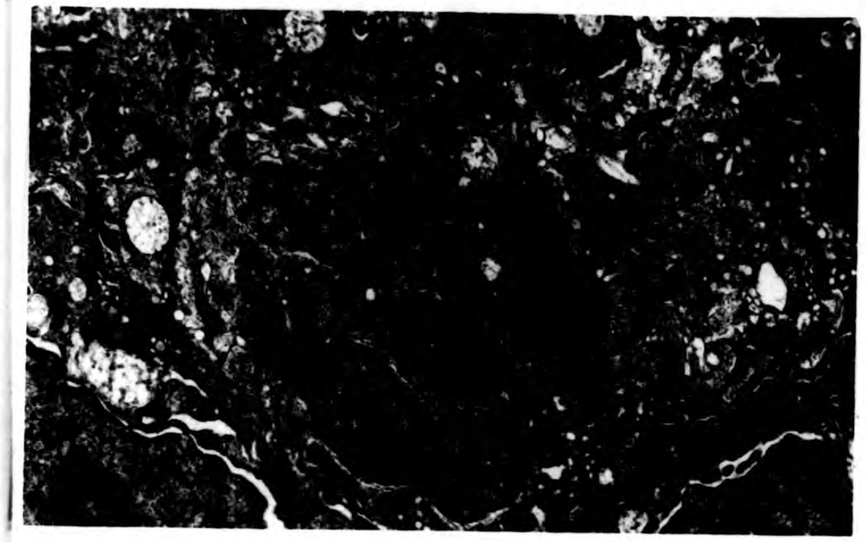


FIGURE 48 - Electron micrograph of juvenile epidermis showing an aggregate of lymphocyte-like cells, which seem to be compressed, their nuclei assuming unusual irregular shapes. This juvenile was vaccinated by direct immersion in a Vibrio anguillarum vaccine. x 11 200.

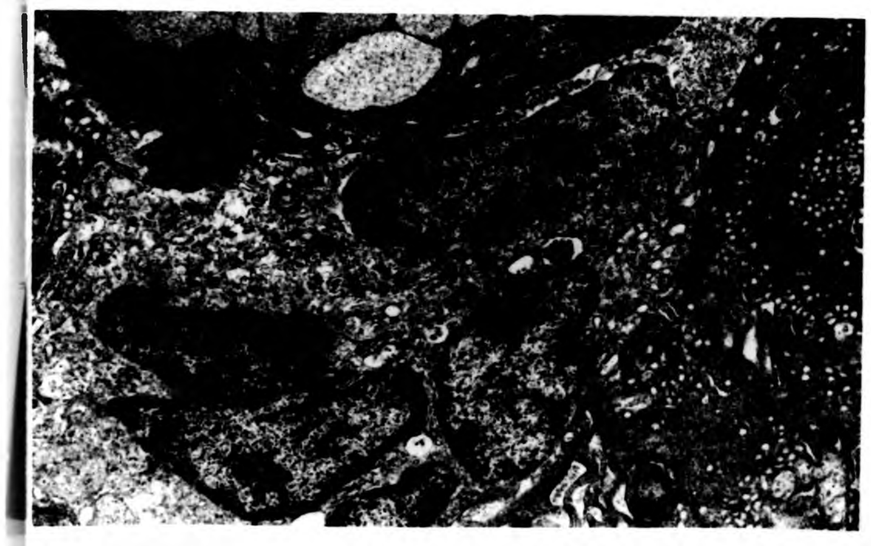
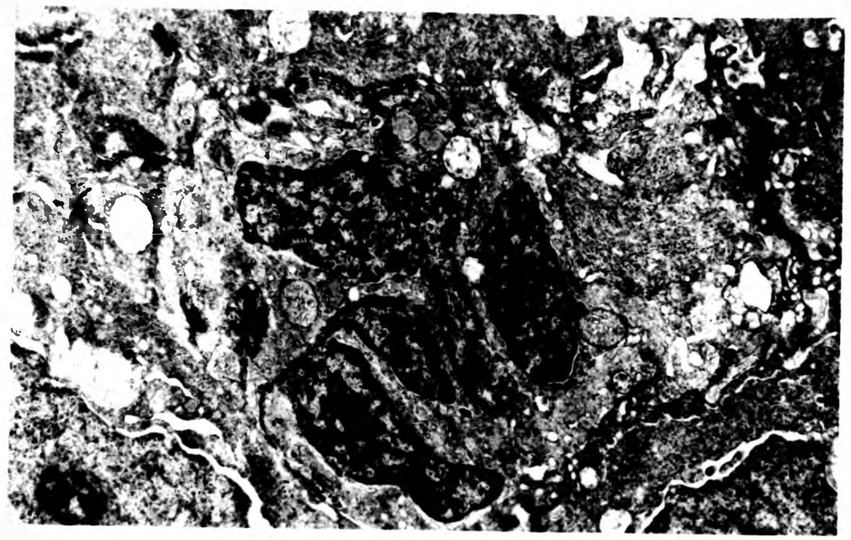


FIGURE 49 - Electron micrograph of juvenile epidermis. Several nuclear lobes (n) of a macrophage-like cell are shown. The cytoplasm is filled with small vesicles and free ribosomes, as well as few mitochondria and a centriole (arrow). A mature mucous cell (M), with rough endoplasmic reticulum (rer), mitochondria and tonofilaments (t), can also be seen. On the right hand side are two filament-containing cells. This juvenile was vaccinated by direct immersion in a Vibrio anguillarum vaccine. x 16 900.



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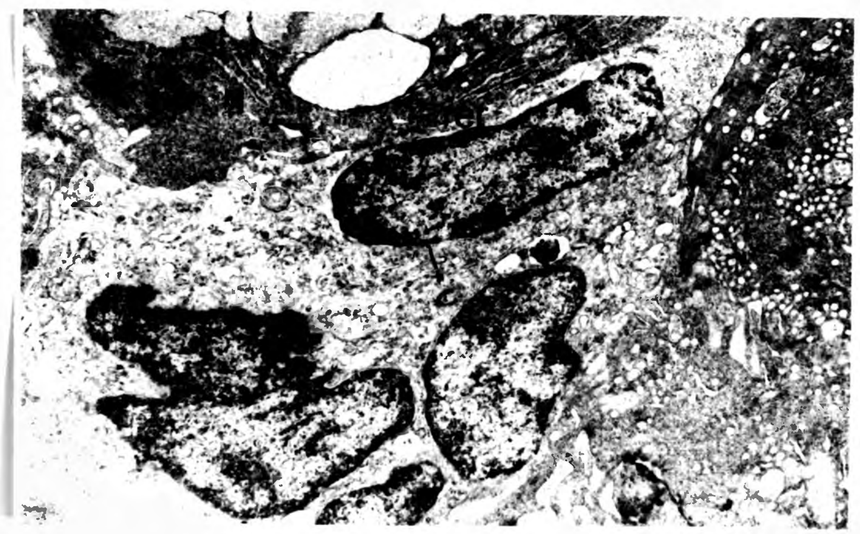

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FIGURE 50 - Electron micrograph of mature female epidermis, showing a cell with a dense lobulated nucleus (n) and a clear cytoplasm, filled with small electron dense bodies, some of which are surrounded by a clear halo (arrowheads). Mitochondria (arrow), rough endoplasmic reticulum (rer) and free ribosomes can also be seen. In the lower left side of the photograph a cytoplasmic extension of the same cell, or of a similar one, is shown. Above it and in close contact with the clear cell is a lymphocyte--like cell (L). A macrophage-like cell (MA) can be seen in the right hand side with a moderately dense nucleus and nucleolus as well as mitochondria and smooth surface elongated vesicles. x 18 000.

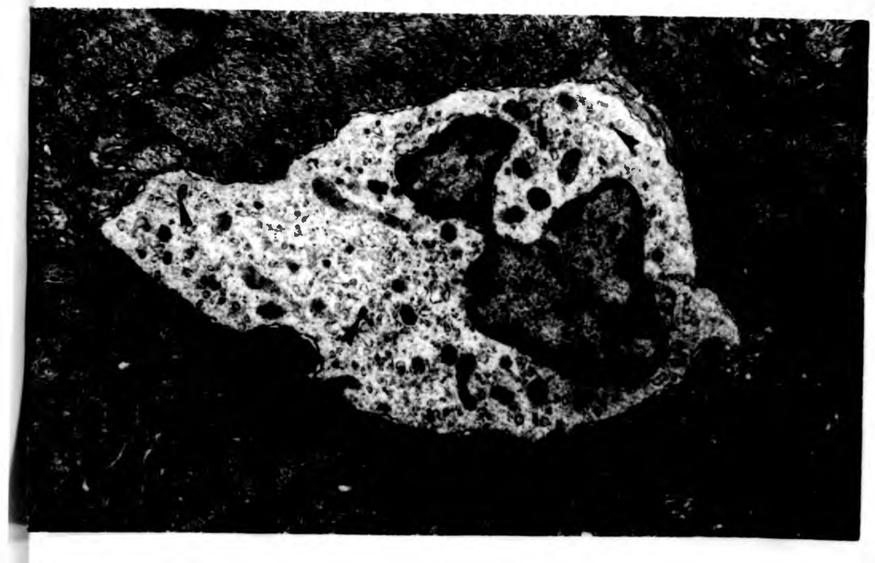

FIGURE 51 - Electron micrograph of mature female epidermis, showing a cell with a deeply clefted nucleus (n) and electron lucent cytoplasm. The chromatin is particularly condensed in the periphery of the nucleus. The cytoplasm shows numerous small vesicles, free ribosomes and a few small dense bodies with a clear halo (arrowheads). Around the cell are filament-containing cells with tonofilaments connected through desmosomes. x 13 800.

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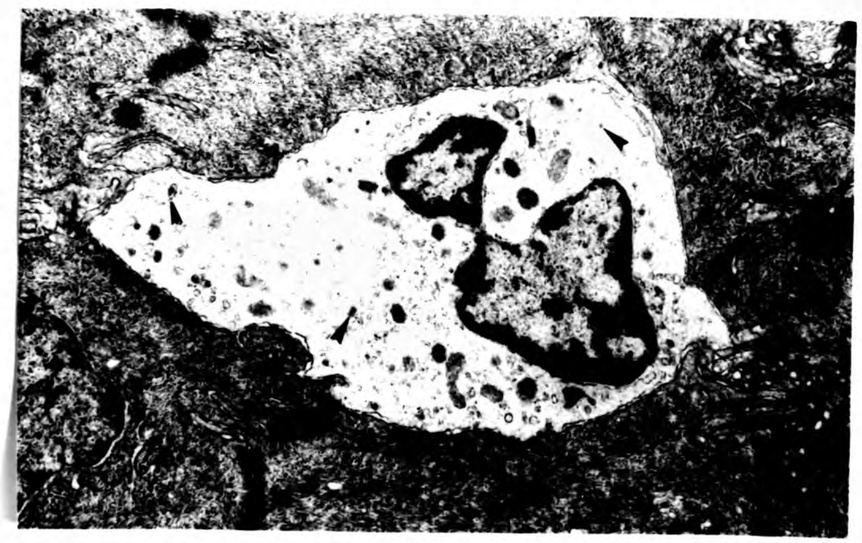

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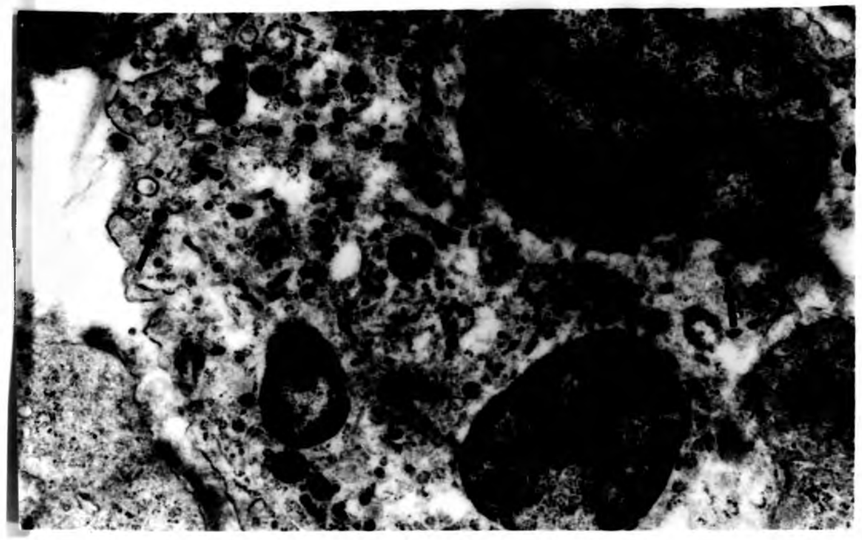

FIGURE 52 - Electron micrograph of mature female epidermis. The internal structure of a cell with electron-lucent cytoplasm can be seen in detail. Note the three nuclear lobes. The electron density and size of the granules is variable. Many of them show a clear surrounding halo (arrows). Free ribosomes and a centriole (c) are also seen. x 17 000.

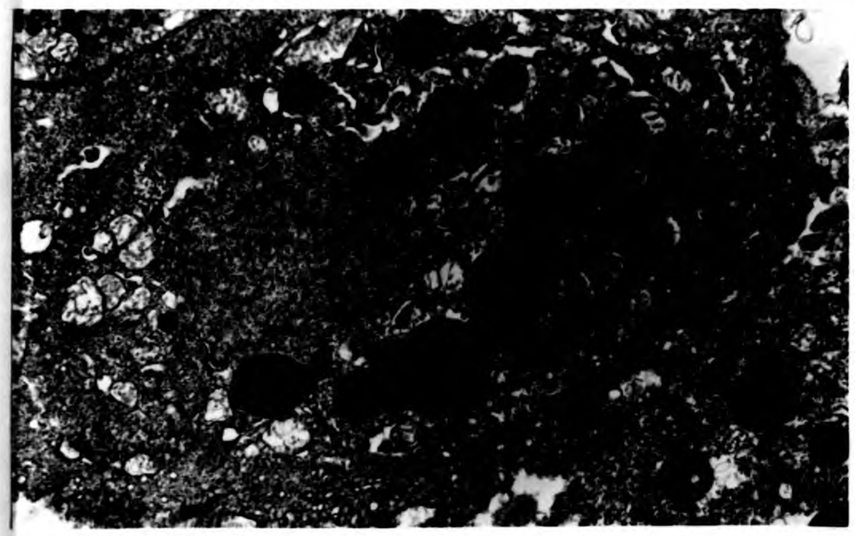

FIGURE 53 - Electron micrograph of mature female epidermis, showing a filament--containing cell with large dense bodies in the perinuclear area. Desmosomes (arrows) are formed with a neighbouring cell, which shows the same electron dense bodies. Note the decrease in tonofilaments found in these cells, whenever compared with normal ones. Nucleus (n). x 7 600. A magnification of the marked area (*) is shown in figure 54.

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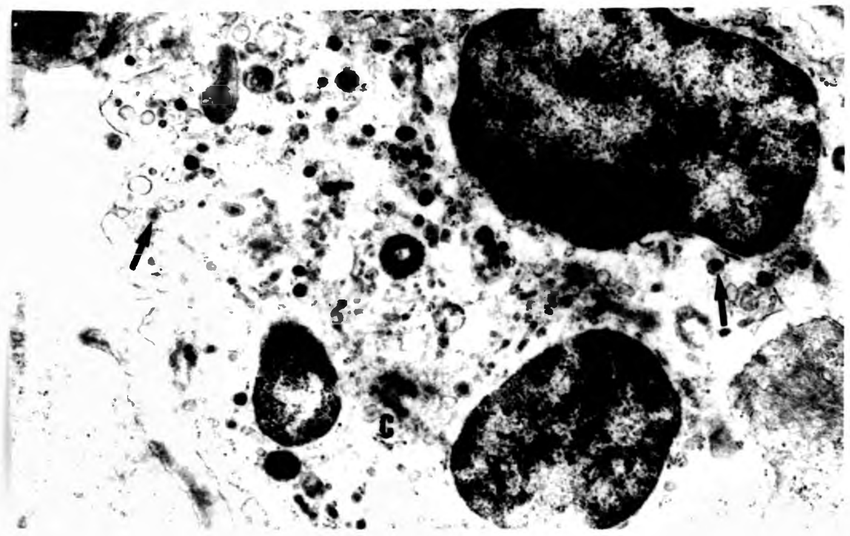

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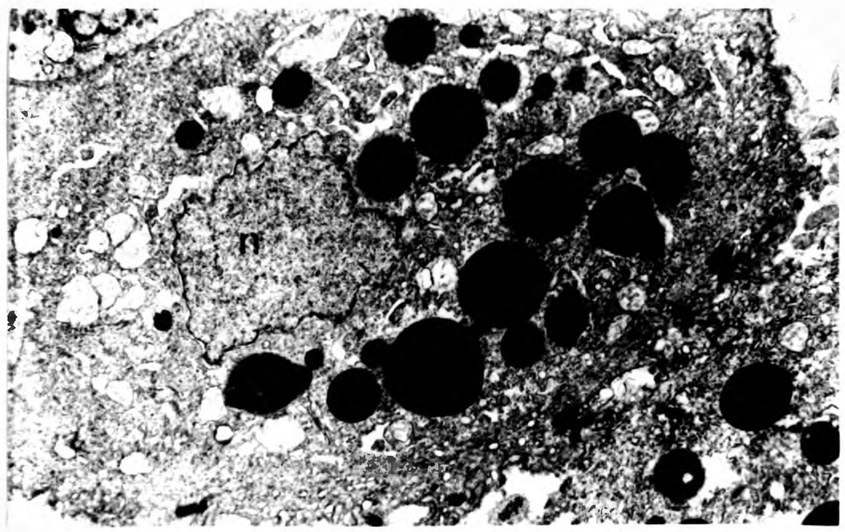

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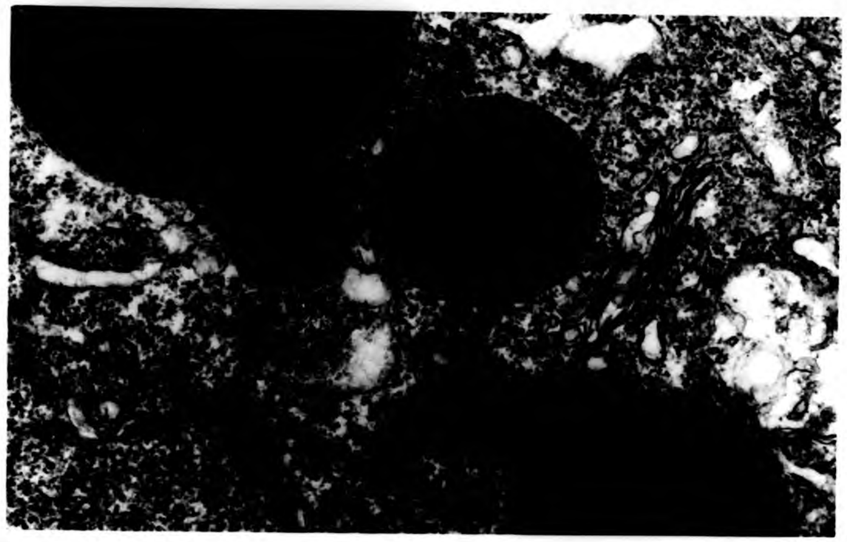

FIGURE 54 - Magnification of the marked area in figure 53, showing the finely fibrillar structure of the dense bodies, limited by a single unit membrane. Two of them (arrows) contain small vesicles, with similar density to the matrix of the dense bodies. x 30 000.

FIGURE 55 - Electron micrograph of mature female epidermis, showing large vacuoles of heterogenous content in the cytoplasm of a filament-containing cell. The nucleus (n) can be seen in the upper side. Note the Golgi saccules (g), the rough endoplasmic reticulum and free ribosomes. Inside the vacuoles, membranous as well as amorphous material of variable density is seen. x 36 000.

FIGURE 54 - Magnification of the marked area in figure 53, showing the finely fibrillar structure of the dense bodies, limited by a single unit membrane. Two of them (arrows) contain small vesicles, with similar density to the matrix of the dense bodies. x 30 000.

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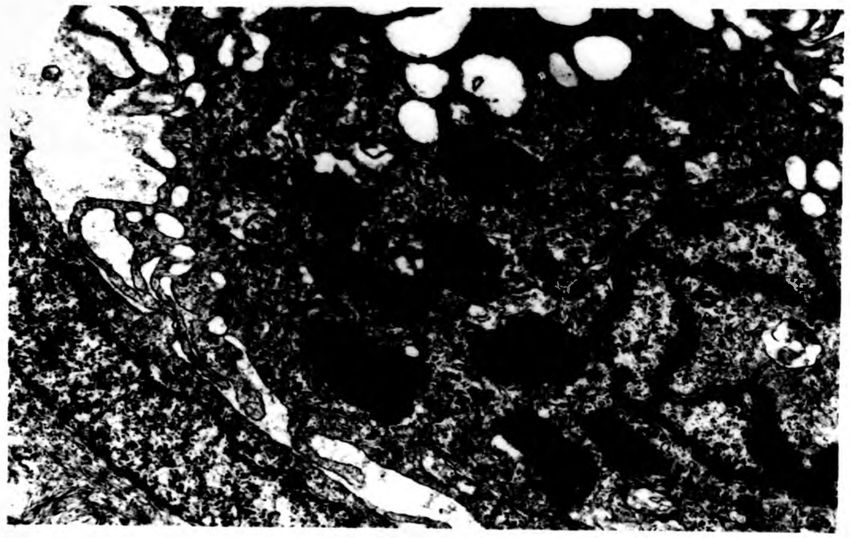


FIGURE 56 - Electron micrograph of mature female epidermis. A macrophage-like cell is shown with an extremely irregular outline. The nucleus is deeply clefted and a few vacuoles (v) of heterogenous content are seen in the cytoplasm. Other structures are mitochondria, rough endoplasmic reticulum and free ribosomes. Small lysosome-like bodies can be seen (arrowheads). x 24 500.

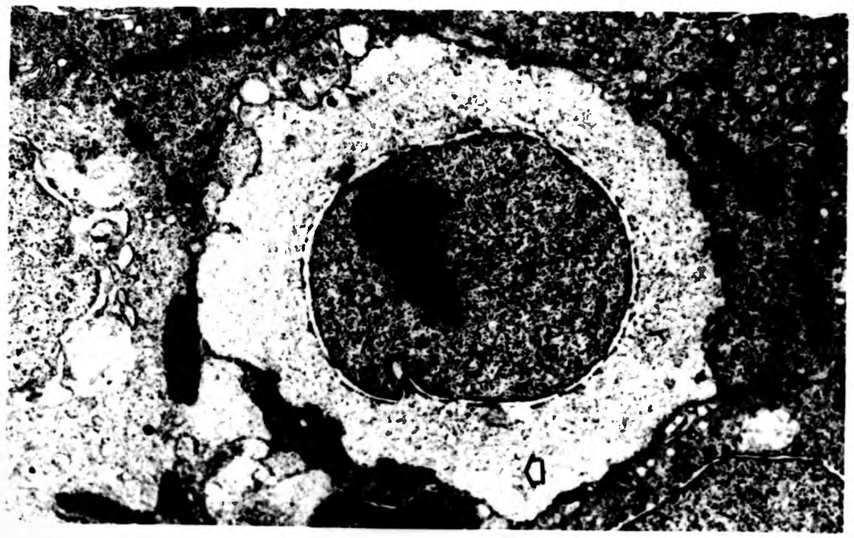
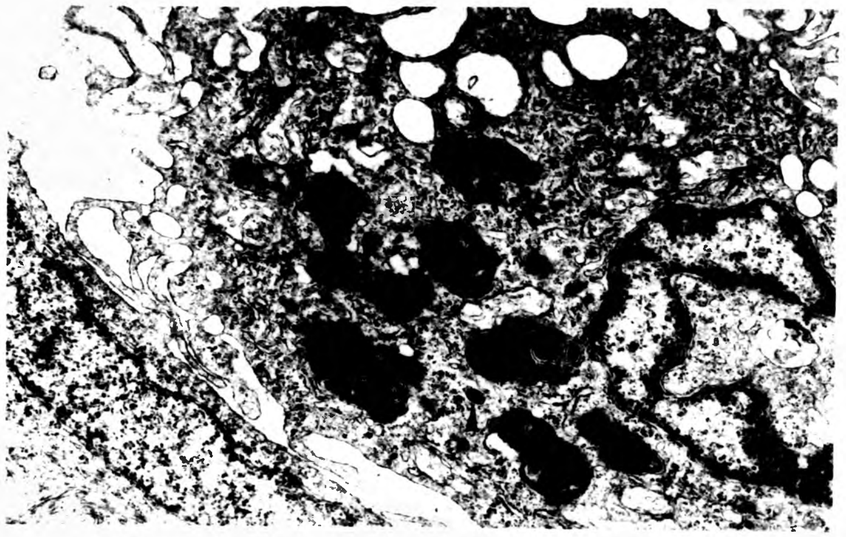


FIGURE 57 - Electron micrograph of juvenile epidermis, showing a small cell with electron lucent cytoplasm and finely granular nuclear chromatin. The nucleolus is very dense. Note the paucity of cytoplasmic inclusions, the whole volume being occupied by filaments, free ribosomes and occasionally electron lucent vesicles (arrows). Around the cell are filament-containing cells and desmosomal junctions. x 10 600.



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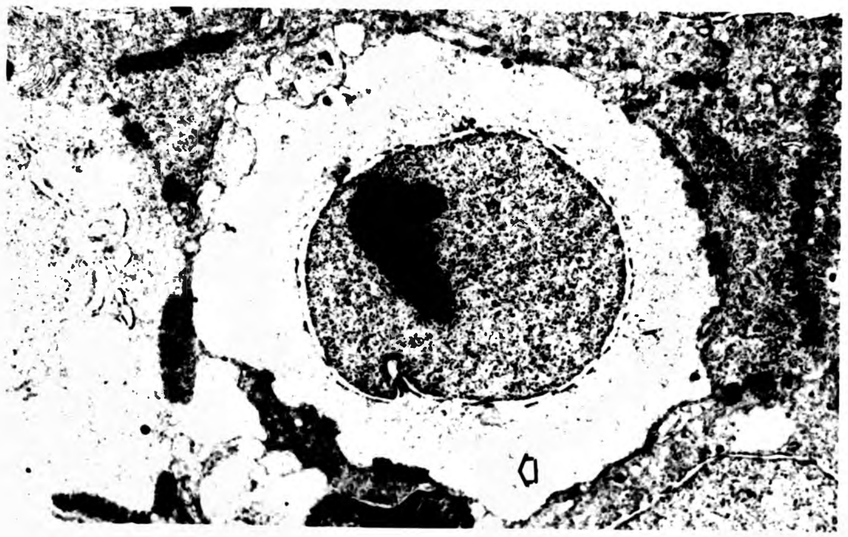

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n

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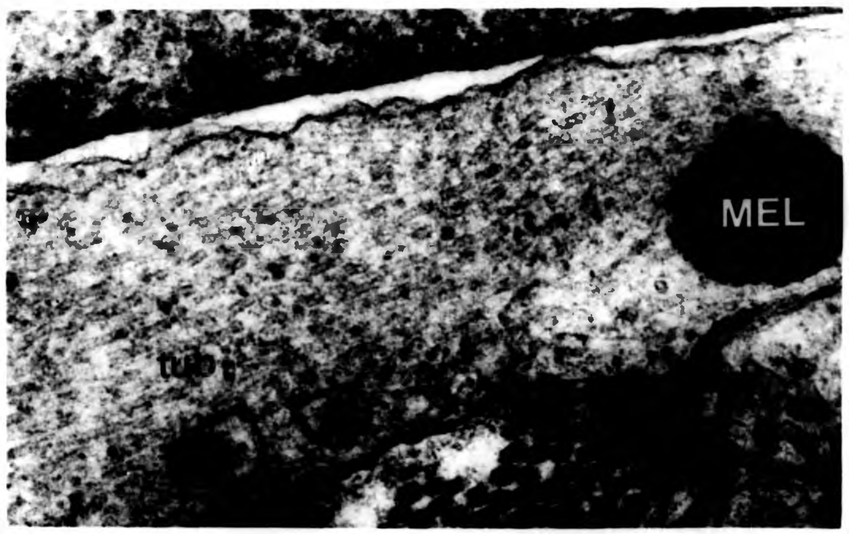
FIGURE 58 - Electron micrograph of a section of mature female skin. The dermis just underneath the basement membrane (BM) is shown with collagen bundles (col), in longitudinal and transverse section, and a melanocyte. Note the elongated nucleus (n) and the numerous electron dense melanosomes in the cytoplasm. A magnification of the marked area (*) is shown in figure 59. x 7 600.

FIGURE 59 - Magnification of figure 58, showing a melanosome (MEL), mitochondria (m) and microtubules (tub). Transverse sections of collagen fibres (col) can be seen outside the cell. x 100 000.



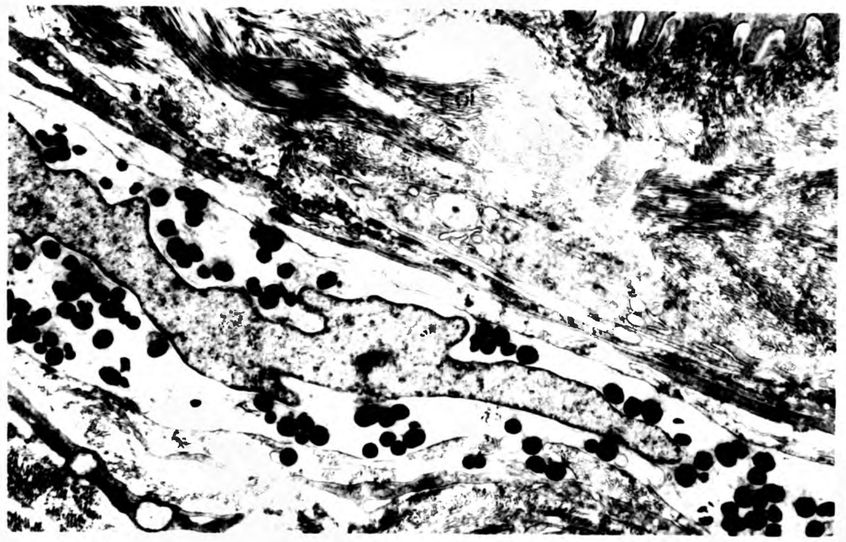


FIGURE 58 - Electron micrograph of a section of mature female skin. The dermis just underneath the basement membrane (BM) is shown with collagen bundles (col), in longitudinal and transverse section, and a melanocyte. Note the elongated nucleus (n) and the numerous electron dense melanosomes in the cytoplasm. A magnification of the marked area (*) is shown in figure 59. x 7 600

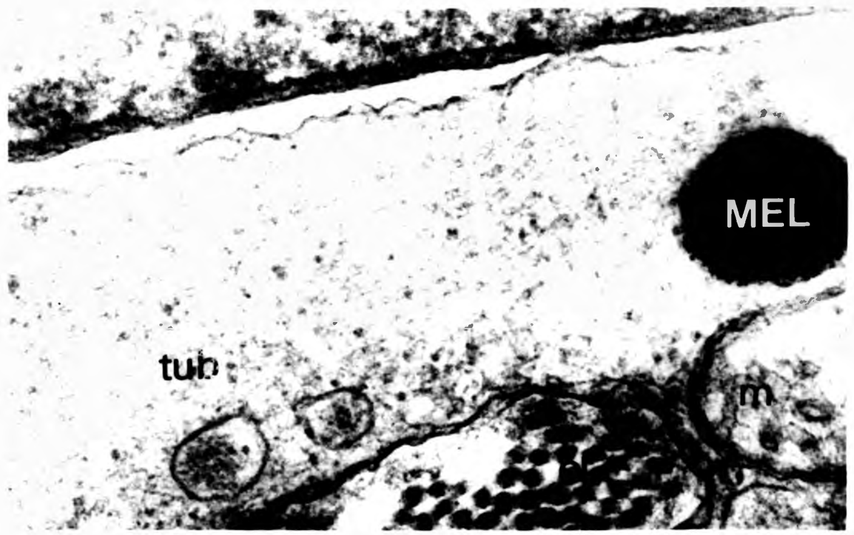
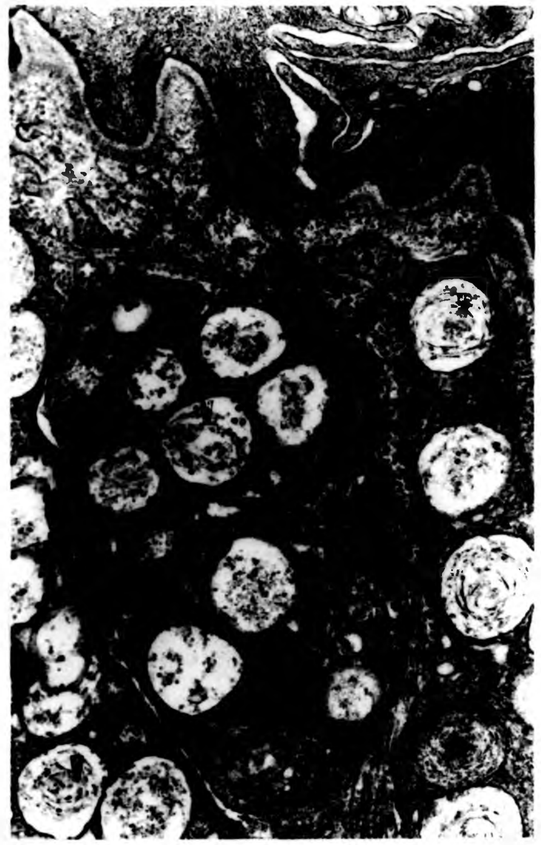
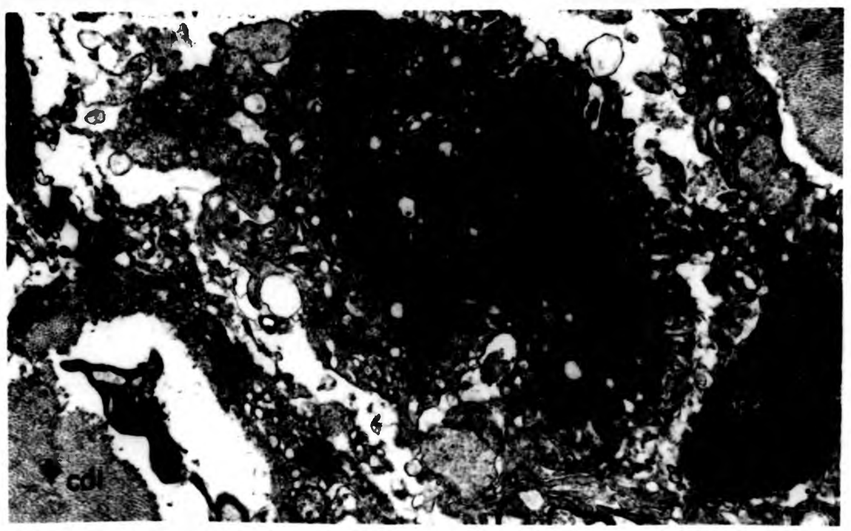


FIGURE 59 - Magnification of figure 58, showing a melanosome (MEL), mitochondria (m) and microtubules (tub). Transverse sections of collagen fibres (col) can be seen outside the cell. x 100 000.

FIGURE 60 - Electron micrograph of mature female skin, showing the cytoplasmic processes of a melanocyte just underneath the basement membrane. The processes are filled with electron lucent vesicles (v) which contain thin fibrils concentrically disposed, interspersed with small granules and amorphous material. x 15 000.

FIGURE 61 - Electron micrograph of a mature female skin. A dermal macrophage is shown with a dense nucleus and many dense bodies, some of which seem to be phagosomes with phagocytosed melanin granules (arrows). Other internal structures are mitochondria and Golgi saccules (g). The remaining melanin granules are inside the cytoplasmic processes of a melanocyte (arrowheads). Collagen bundles (col).
x 10 000.





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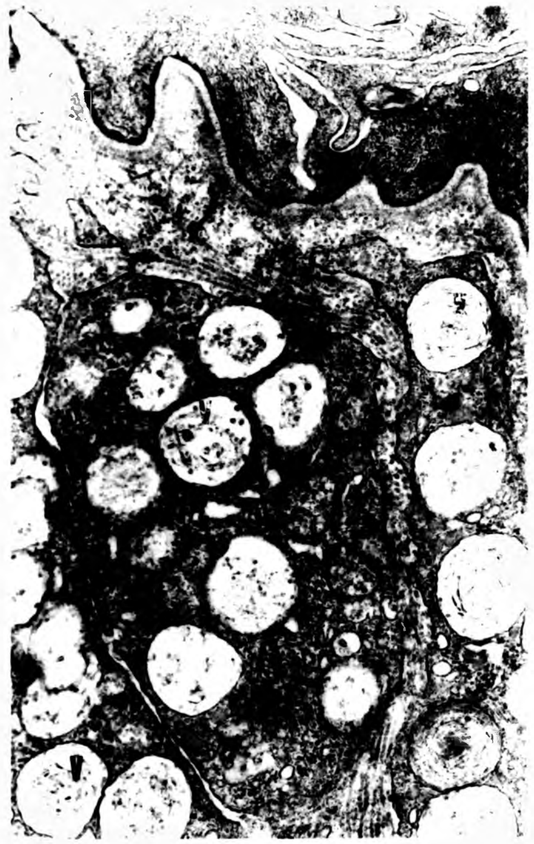

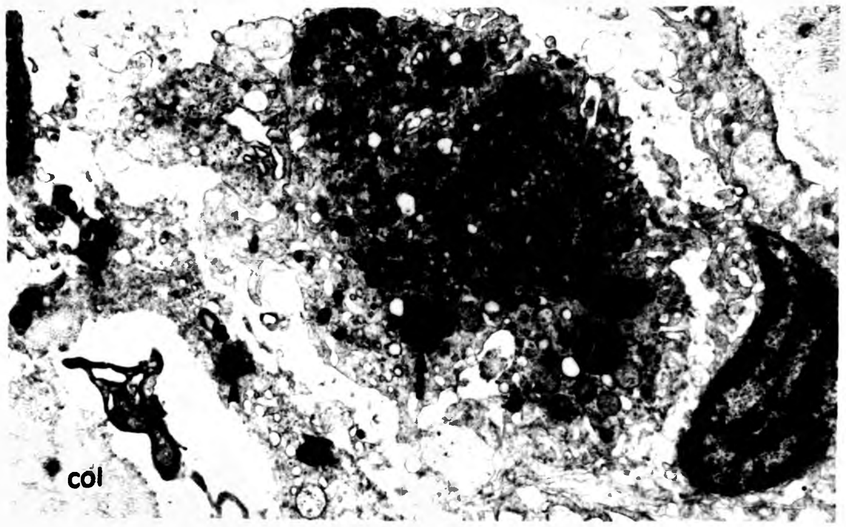

FIGURE 62 - Electron micrograph of mature female epidermis. The cytoplasm of a macrophage is shown with numerous phagosomes (PH). These include melanin granules, amorphous material and membranes. In the lower area, part of a lymphocyte (L) is seen. Filament-containing cells surround the other two cells. x 9600.

FIGURE 63 - Electron micrograph of juvenile skin. Basal filament-containing cells are shown, one of which carries melanin granules in its cytoplasm, just above a group of mitochondria (m). The basement membrane (BM) is seen in the lower right corner. Note the thin interdigitating folds of the cell membranes contrasting with the low electron dense background. This juvenile was used as control in the vaccination experiments. x 9600.

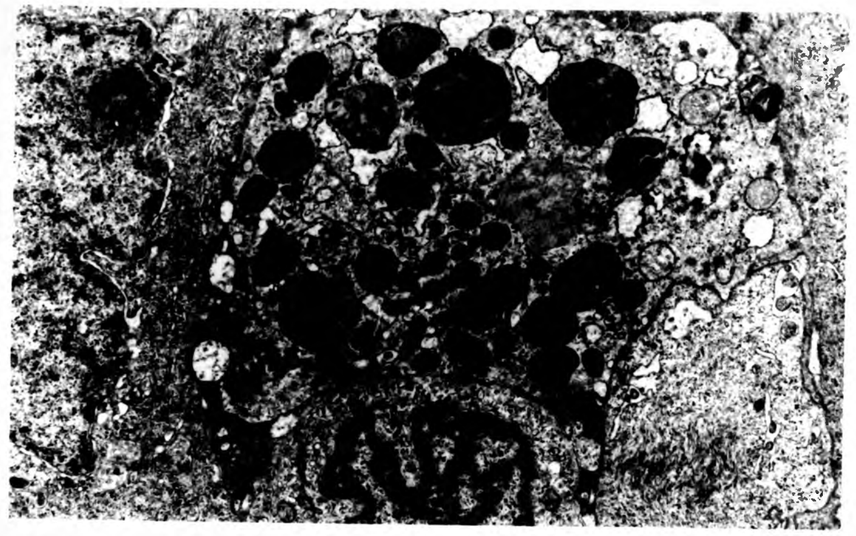

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FIGURE 64 - Electron micrograph of juvenile skin, showing a melanin-containing cell moving through the basement membrane. Epidermis (E); dermis (D). Note the thin reticular fibres just underneath the basal lamina. x 12 000.

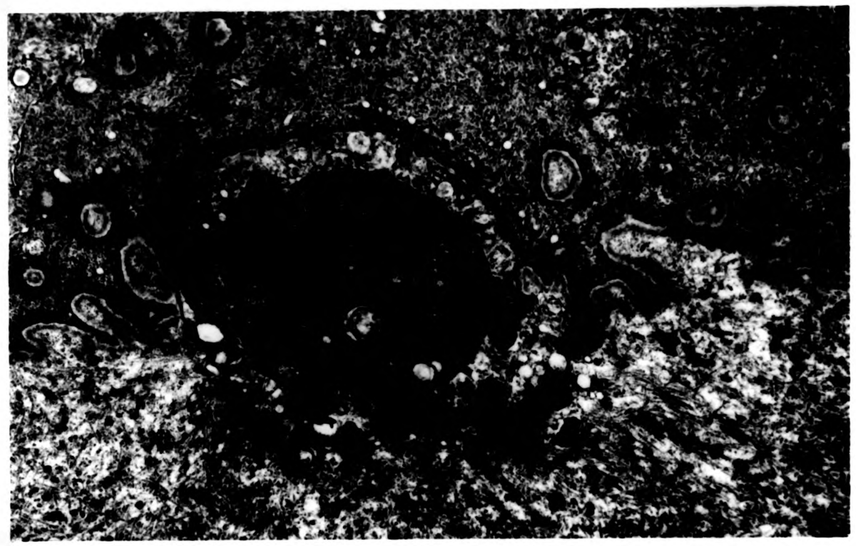
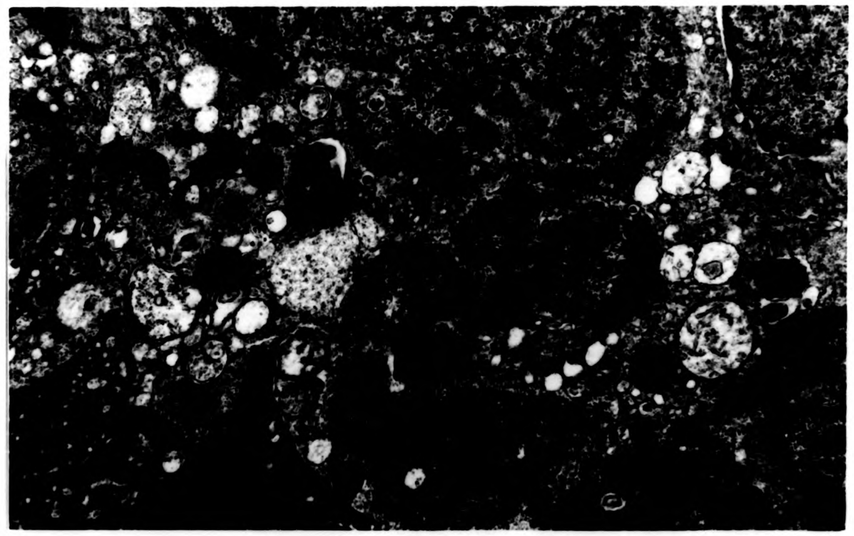
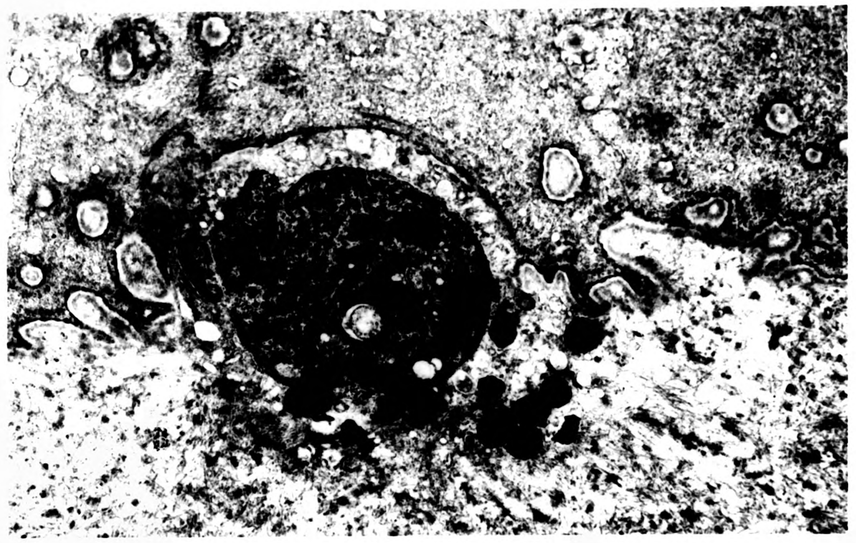


FIGURE 65 - Electron micrograph of mature female epidermis, showing a melanin-containing cell with a few irregular masses of nuclear material (n) and melanin granules. The cell also shows cytoplasmic processes between the neighbouring filament-containing cells (FCC). x 13 000.





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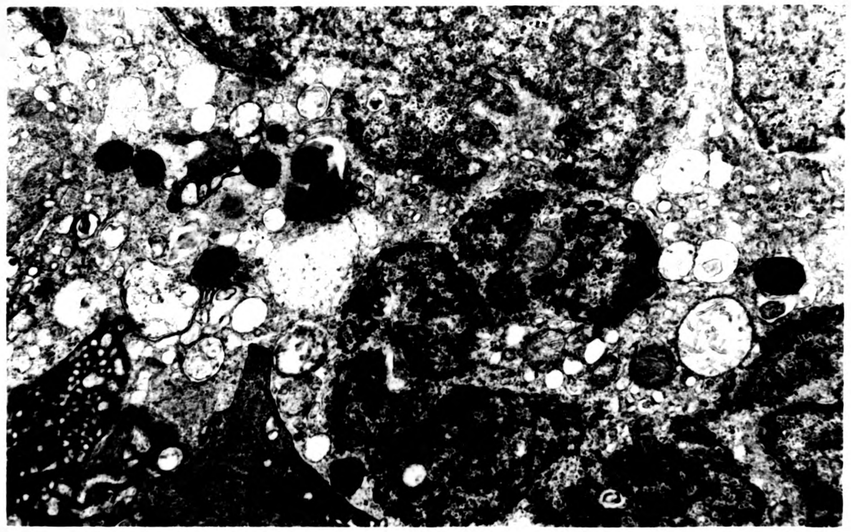


FIGURE 65 - Electron micrograph of mature female epidermis, showing a melanin-containing cell with a few irregular masses of nuclear material (n) and melanin granules. The cell also shows cytoplasmic processes between the neighbouring filament-containing cells (FCC). x 13 000.

FIGURE 66 - Electron micrograph of mature female dermis, showing part of two fibroblasts with elongated nucleus (n), rough endoplasmic reticulum (rer), mitochondria (m) and numerous small vesicles. Collagen fibres (col) are seen in longitudinal and transverse section around the cells. x 15 600.

FIGURE 67 - Electron micrograph of a mature female dermis. A myelinated nerve fibre is shown with a thick dense myelin sheath (m). x 16 000.

FIGURE 66 - Electron micrograph of mature female dermis, showing part of two fibroblasts with elongated nucleus (n), rough endoplasmic reticulum (rer), mitochondria (m) and numerous small vesicles. Collagen fibres (col) are seen in longitudinal and transverse section around the cells. x 15 600.

FIGURE 67 - Electron micrograph of a mature female dermis. A myelinated nerve fibre is shown with a thick dense myelin sheath (m). x 16 000.

FIGURE 68 - Electron micrograph of mature female dermis, showing an iridophore with its typical plate-clefts (PC). x 15 200.

FIGURE 69 - Electron micrograph of a mature female skin. The upper dermis is shown with collagen bundles (col) just underneath the basement membrane (BM). Note the dermal infiltration with monocyte-like cells, with dense nucleus and cytoplasm containing numerous electron dense granules with a surrounding pale halo (arrows). Mitochondria and rough endoplasmic reticulum can also be seen. x 13 000.

FIGURE 68 - Electron micrograph of mature female dermis, showing an iridophore with its typical plate-clefts (PC). x 15 200.

FIGURE 69 - Electron micrograph of a mature female skin. The upper dermis is shown with collagen bundles (col) just underneath the basement membrane (BM). Note the dermal infiltration with monocyte-like cells, with dense nucleus and cytoplasm containing numerous electron dense granules with a surrounding pale halo (arrows). Mitochondria and rough endoplasmic reticulum can also be seen. x 13 000.

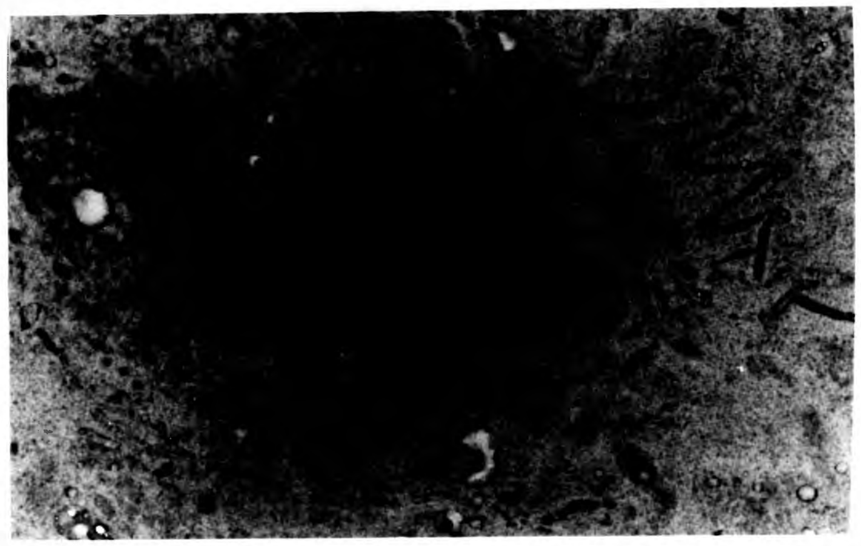


FIGURE 70 - Electron micrograph of mature male epidermis treated with PTA. Observe the discontinuous dense staining of the cell membranes of the filament-containing cells (arrows). Small dense bodies can be seen around the centre of the cell. x 13 000.

FIGURE 71 - Electron micrograph of mature male epidermis treated with PTA. A filament-containing cell is shown,with a characteristic horse shoe shaped nucleus (n). Note the small PTA-positive dense bodies in the cytoplasm. x 13 000.

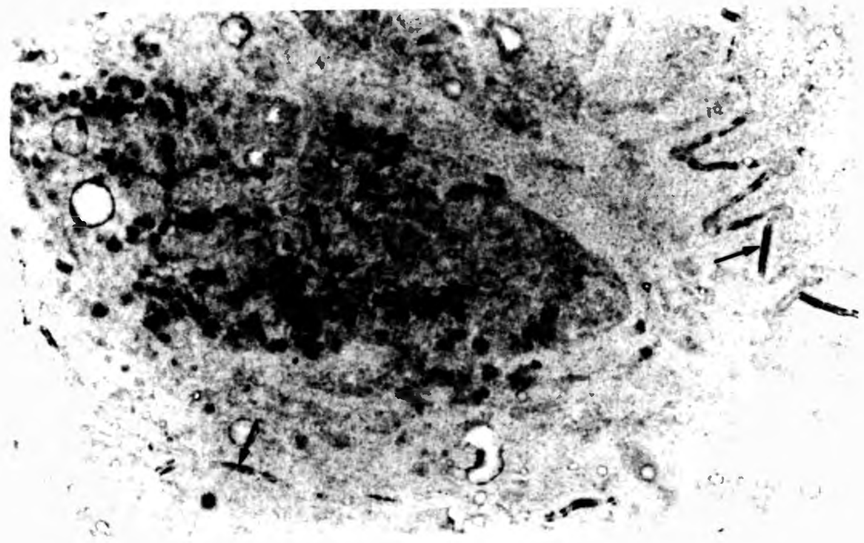


FIGURE 70 - Electron micrograph of mature male epidermis treated with PTA. Observe the discontinuous dense staining of the cell membranes of the filament-containing cells (arrows) Small dense bodies can be seen around the centre of the cell. x 13 000.

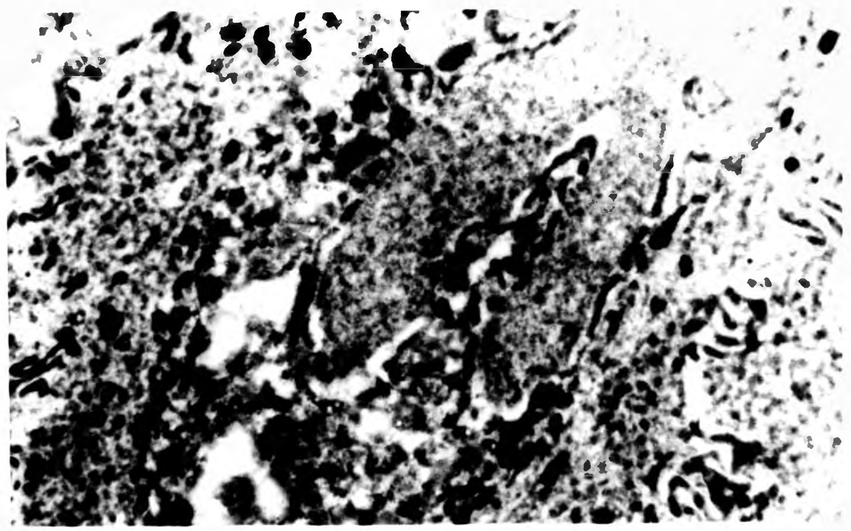


FIGURE 71 - Electron micrograph of mature male epidermis treated with PTA. A filament-containing cell is shown,with a characteristic horse shoe shaped nucleus (n). Note the small PTA-positive dense bodies in the cytoplasm. x 13 000

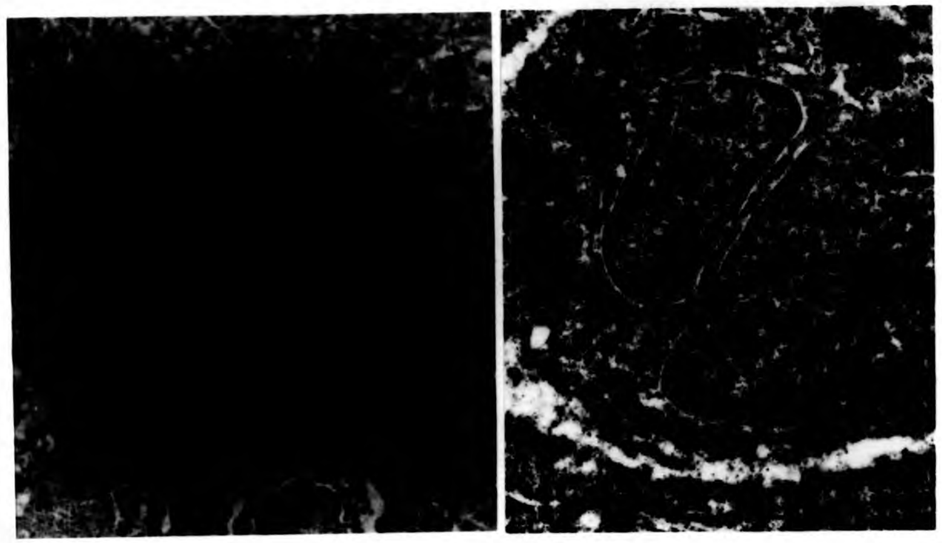


FIGURE 72 - A. Electron micrograph of mature female epidermis treated with PTA, showing two macrophages with large bodies, possibly lysosomes, with PTA-positive membranes. x 11 000. B. Electron micrograph of mature female epidermis. A different area of the same section shown in A, stained conventionally with uranyl acetate and lead citrate (not post-fixed with osmium tetroxide). A macrophage is shown with two moderately dense nuclear lobes and low electron dense large bodies (b), whose membranes are PTA-positive. x 13000.

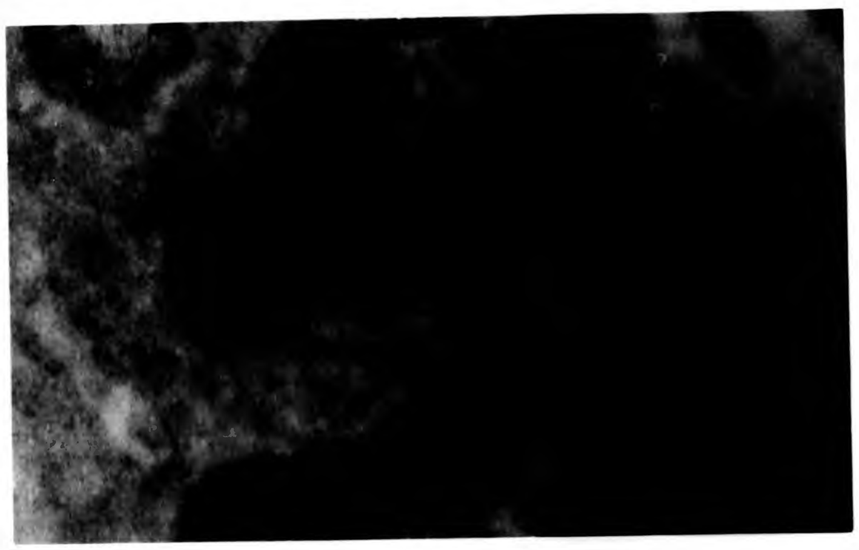


FIGURE 73 - Electron micrograph of mature female epidermis treated with PTA. A limited area of a filament-containing cell is shown, with large bodies (b) which show some PTA-positive material in their membranes. These same bodies can be seen in figure 53, in material conventionally prepared for morphological observations.
x 39 000.

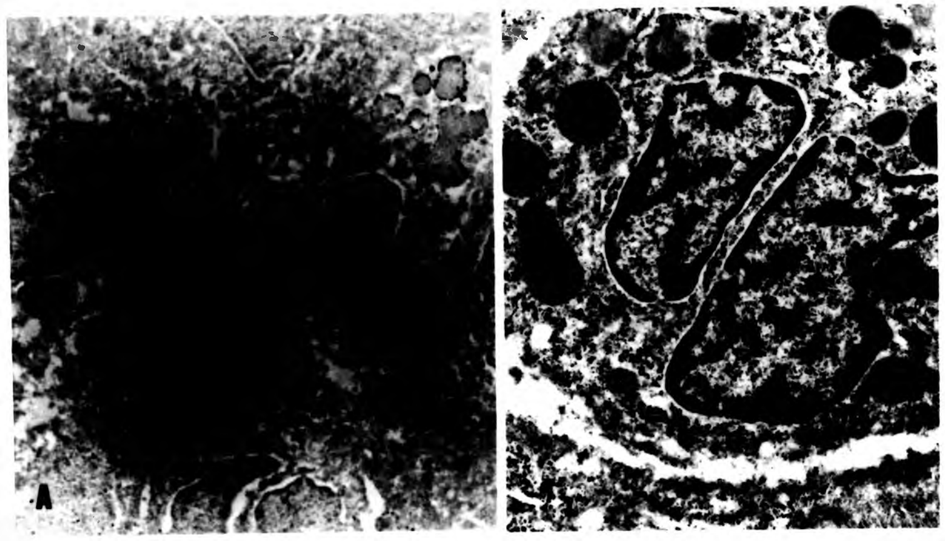


FIGURE 72 - A. Electron micrograph of mature female epidermis treated with PTA, showing two macrophages with large bodies, possibly lysosomes, with PTA-positive membranes. x 11 000. B. Electron micrograph of mature female epidermis. A different area of the same section shown in A, stained conventionally with uranyl acetate and lead citrate (not post-fixed with osmium tetroxide). A macrophage is shown with two moderately dense nuclear lobes and low electron dense large bodies (b), whose membranes are PTA-positive. x 13000.

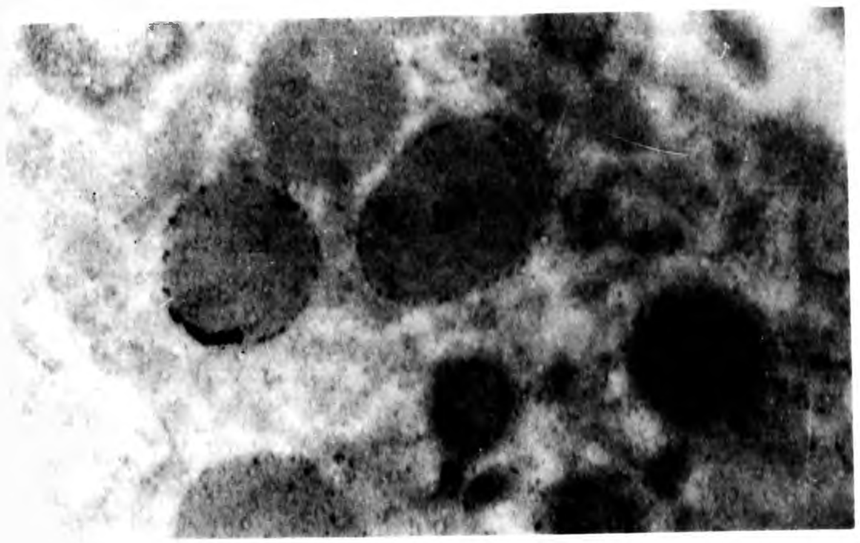


FIGURE 73 - Electron micrograph of mature female epidermis treated with PTA. A limited area of a filament-containing cell is shown, with large bodies (b) which show some PTA-positive material in their membranes. These same bodies can be seen in figure 53, in material conventionally prepared for morphological observations. x 39 000.

FIGURE 74 - Electron micrograph of mature male epidermis treated with PTA, showing a fully developed mucous cell. The staining characteristics of the mucous vesicles are variable. The intensity of the staining is also variable amongst the positive vesicles. No staining can be seen in the rough endoplasmic reticulum (arrows). x 9 000.

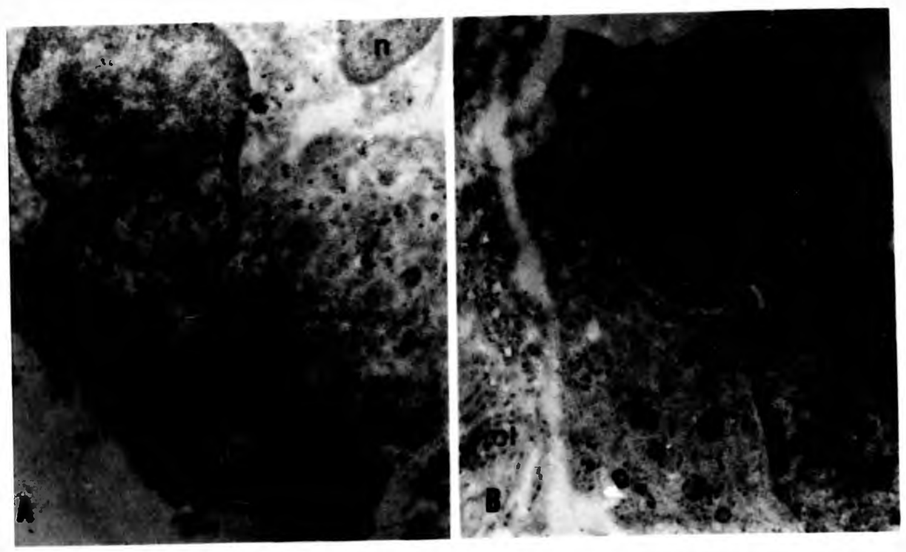


FIGURE 75 - A. Electron micrograph of mature female epidermis treated with PTA. A clear cell with multilobulated nucleus (n) is shown, with small dark granules and vesicles which stained positively with PTA. The fine structure of this cell type can be appreciated in figures 50, 51 and 52. x 17 000. B. Electron micrograph of mature female skin treated with PTA. A dermal macrophage is shown, containing large bodies with PTA-positive membranes. Collagen fibres (col) can be seen around the cell. x 17 000.

FIGURE 74 - Electron micrograph of mature male epidermis treated with PTA, showing a fully developed mucous cell. The staining characteristics of the mucous vesicles are variable. The intensity of the staining is also variable amongst the positive vesicles. No staining can be seen in the rough endoplasmic reticulum (arrows). x 9 000.

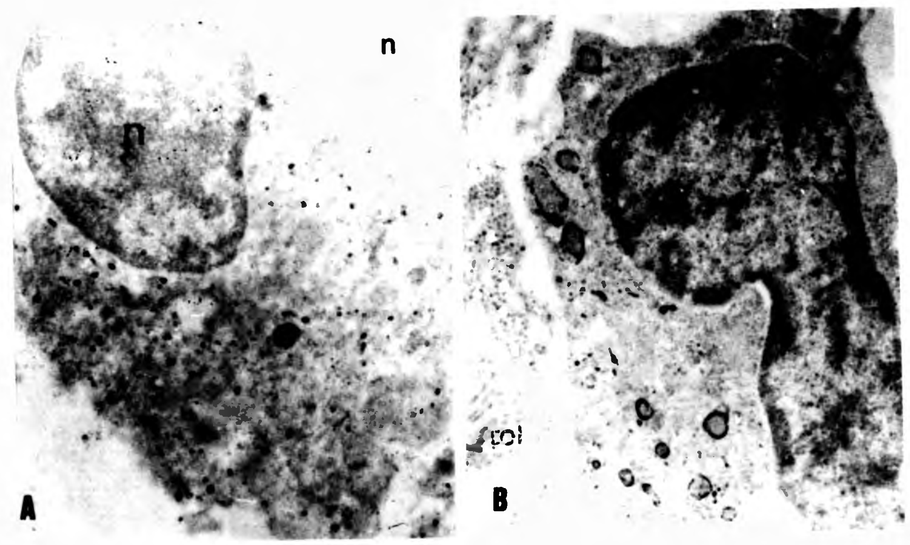


FIGURE 75 - A. Electron micrograph of mature female epidermis treated with PTA. A clear cell with multilobulated nucleus (n) is shown, with small dark granules and vesicles which stained positively with PTA. The fine structure of this cell type can be appreciated in figures 50, 51 and 52. x 17 000. B. Electron micrograph of mature female skin treated with PTA. A dermal macrophage is shown, containing large bodies with PTA-positive membranes. Collagen fibres (col) can be seen around the cell. x 17 000.

A

B

C

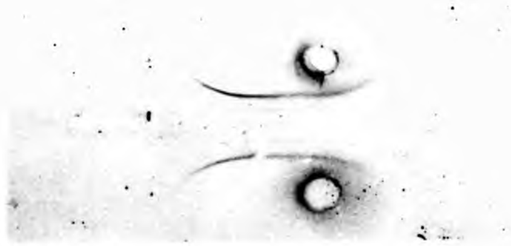


FIGURE 76 - Immunoelectrophoresis of the rabbit serum anti-trout IgM against whole trout serum. A and C - trout serum. B - rabbit serum anti-trout IgM. The catode is to the right.

A

B

C

FIGURE 77 - Immunoelectrophoresis of the rabbit serum anti-whole trout serum against trout serum. A and C - trout serum. B - rabbit serum anti-whole trout serum. The catode is to the right.

FIGURE 78 - Immunoelectrophoresis of the rabbit serum anti-whole trout serum, against the first eluted fraction obtained through chromatography on Sephadex G-200 of the ammonium sulphate precipitate of trout serum. A and C - rabbit serum anti--whole trout serum. B - first eluted fraction. The catode is to the right.

A

B

C

FIGURE 76 - Immunoelectrophoresis of the rabbit serum anti-trout IgM against whole trout serum A and C - trout serum. B - rabbit serum anti-trout IgM. The catode is to the right.

FIGURE 77 - Immunoelectrophoresis of the rabbit serum anti-whole trout serum against trout serum. A and C - trout serum. B - rabbit serum anti-whole trout serum. The catode is to the right.

FIGURE 78 - Immunoelectrophoresis of the rabbit serum anti-whole trout serum, against the first eluted fraction obtained through chromatography on Sephadex G-200 of the ammonium sulphate precipitate of trout serum. A and C - rabbit serum anti--whole trout serum. B - first eluted fraction. The catode is to the right.

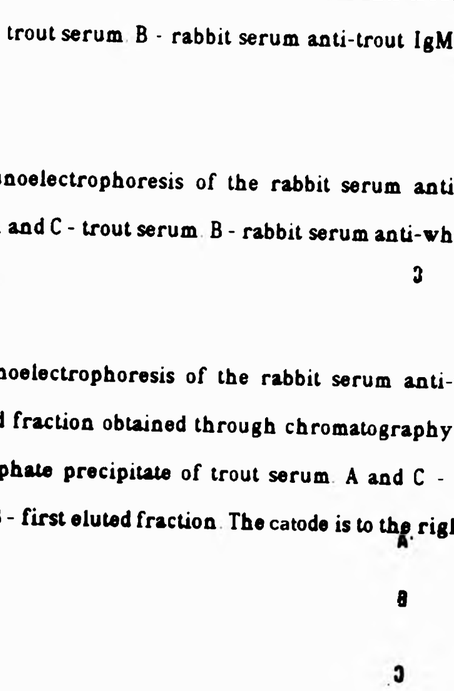
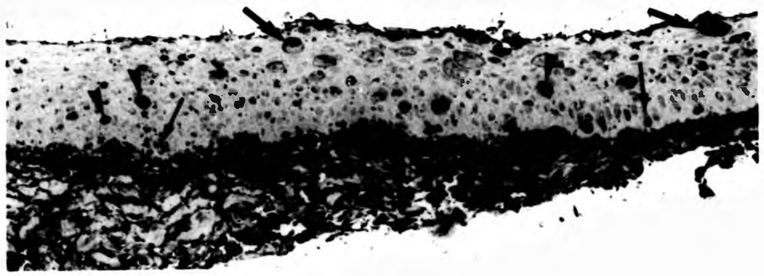


FIGURE 79 - Semithin section of skin of sexually mature rainbow trout female, stained for immunoglobulin (Ig) with the PAP method. The concentration of the rabbit serum anti- trout IgM was 1: 50. Small positive cells can be seen in the epidermis (arrowheads). The mucous cells look either unstained or partially stained (broad arrows). A few malpighian cells show a moderate degree of positive staining (thin arrows). Haematoxylin (H). x 250.

FIGURE 80 - Similar section as in figure 79, stained with the PAP method for the presence of Ig. The concentration of the rabbit serum anti-trout IgM was 1: 800. The reaction is still positive for small cells in the epidermis (arrowheads), and for partially stained mucous cells (arrow). The malpighian cells did not show positive staining at this dilution. H. x 250.

FIGURE 81 - Semithin section of skin of sexually mature rainbow trout female stained for Ig with the PAP method. Normal rabbit serum was used in replacement of the rabbit serum anti-trout IgM. H. x 250.



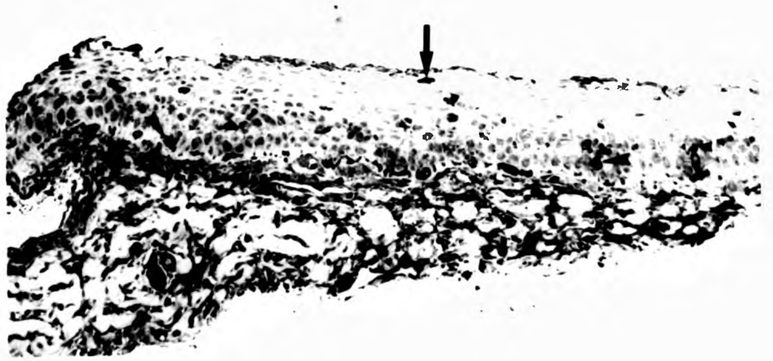


FIGURE 79 - Semithin section of skin of sexually mature rainbow trout female, stained for immunoglobulin (Ig) with the PAP method. The concentration of the rabbit serum anti trout IgM was 1: 50. Small positive cells can be seen in the epidermis (arrowheads). The mucous cells look either unstained or partially stained (broad arrows). A few malpighian cells show a moderate degree of positive staining (thin arrows). Haematoxylin (H) x 250.

FIGURE 80 - Similar section as in figure 79, stained with the PAP method for the presence of Ig. The concentration of the rabbit serum anti-trout IgM was 1: 800. The reaction is still positive for small cells in the epidermis (arrowheads), and for partially stained mucous cells (arrow). The malpighian cells did not show positive staining at this dilution. H. x 250.

FIGURE 81 - Semithin section of skin of sexually mature rainbow trout female stained for Ig with the PAP method. Normal rabbit serum was used in replacement of the rabbit serum anti-trout IgM. H. x 250.

FIGURE 82 - Semithin section of skin of sexually mature rainbow trout male stained for Ig, with the PAP method. Small positive cells can be seen in the epidermis and dermis (arrowheads). The mucous cells show a variable degree of staining. Intercellular staining is noticeable in the epidermis. H. x 250.

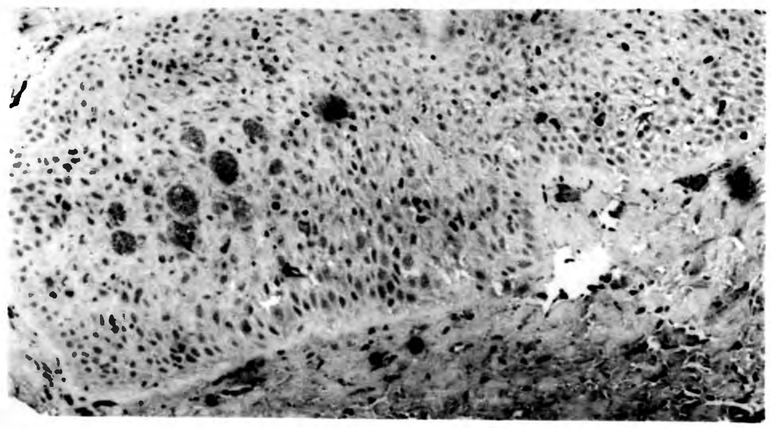


FIGURE 83 - Same section of figure 82 stained with the PAP method, using the rabbit serum anti- trout IgM absorbed with Ig. Note the negative staining both in epidermis and dermis. H. x 250.

FIGURE 82 - Semithin section of skin of sexually mature rainbow trout male stained for Ig, with the PAP method. Small positive cells can be seen in the epidermis and dermis (arrowheads). The mucous cells show a variable degree of staining. Intercellular staining is noticeable in the epidermis. H. x 250.

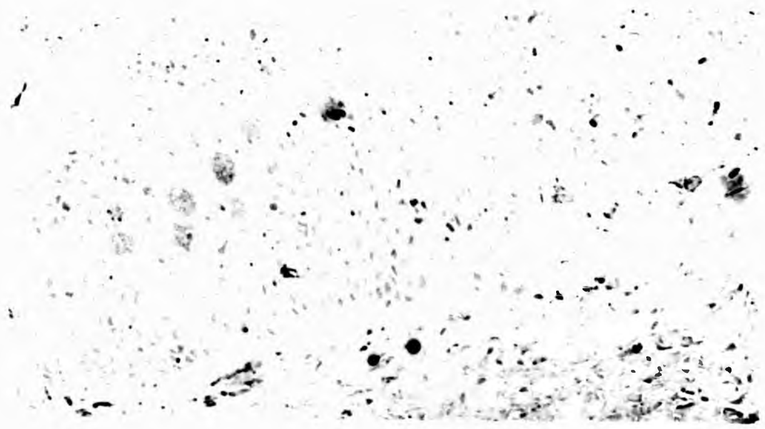
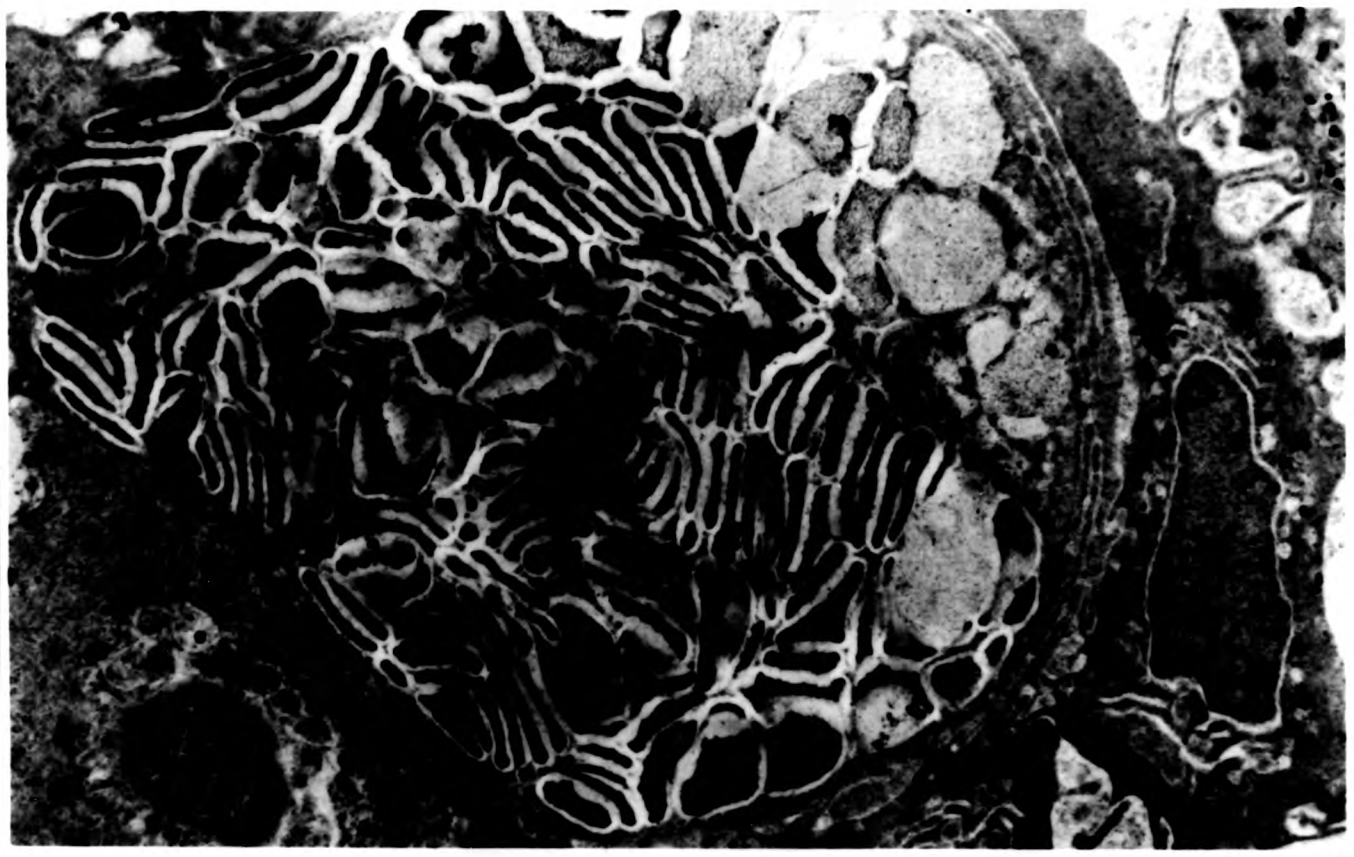


FIGURE 83 - Same section of figure 82 stained with the PAP method, using the rabbit serum anti- trout IgM absorbed with Ig. Note the negative staining both in epidermis and dermis. H. x 250.

FIGURE 84 - Electron micrograph of a mature female epidermis, fixed with Karnovsky, embedded in Epon-Araldite and treated with rabbit serum anti-trout IgM and the PAP method. The section was not stained. Notice the labelled mucous vesicles of a goblet cell. Dark granules can also be seen in the cytoplasm of filament-containing cells (FCC). x 6 520.



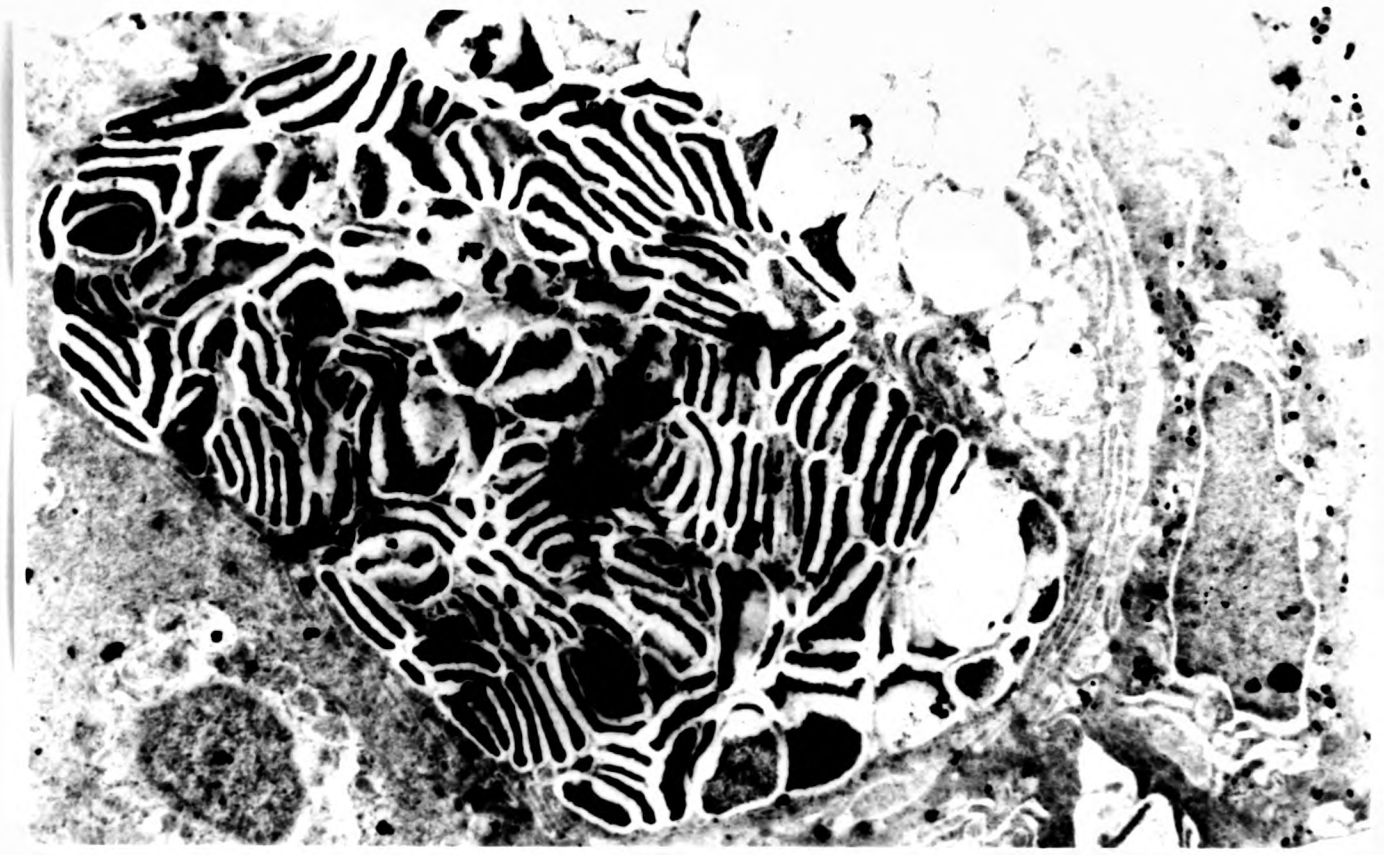
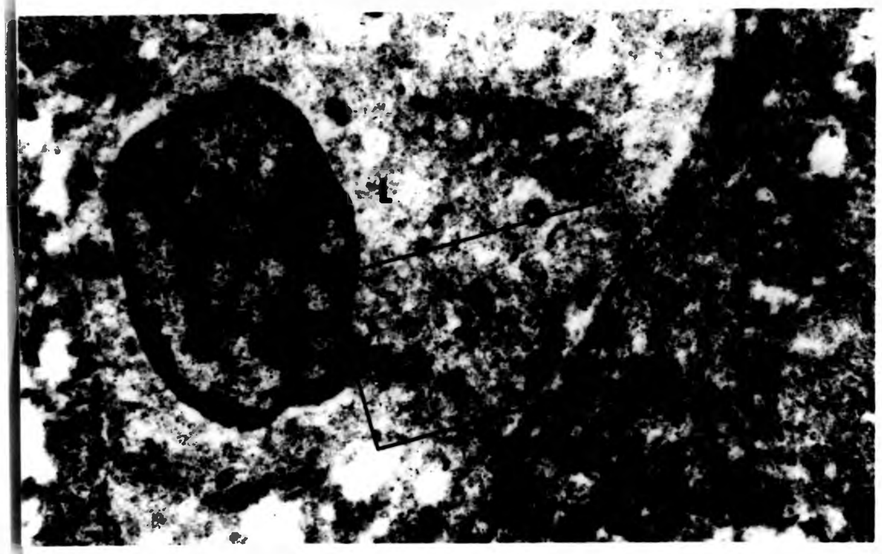
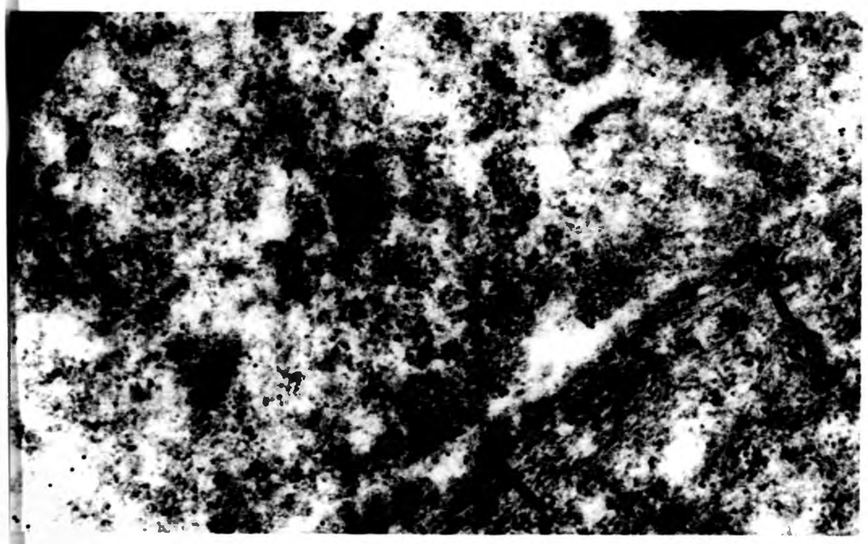


FIGURE 85 - Electron micrograph of a mature female epidermis, fixed with Karnovsky, embedded in Lowicryl and treated with rabbit serum anti-trout IgM and GAR-10. Notice the label over the cytoplasm of a lymphocyte-like cell (L). A magnification of the limited area in the micrograph is shown in figure 86. Adjacent to the lymphocyte-like cell is a filament-containing cell (FCC). x 19 200.

FIGURE 86 - Magnification of the limited area in figure 85. Notice the gold particles which are much more numerous on the cytoplasm of the lymphocyte-like cell. The separation between both cells is ill defined (arrows). x 47 400.





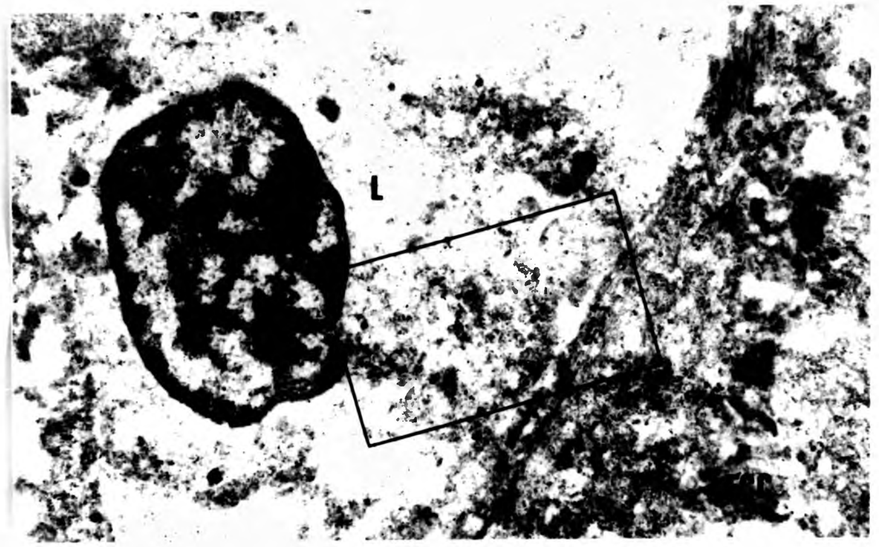


FIGURE 85 - Electron micrograph of a mature female epidermis, fixed with Karnovsky, embedded in Lowicryl and treated with rabbit serum anti-trout IgM and GAR-10. Notice the label over the cytoplasm of a lymphocyte-like cell (L). A magnification of the limited area in the micrograph is shown in figure 86. Adjacent to the lymphocyte-like cell is a filament-containing cell (FCC). x 19 200

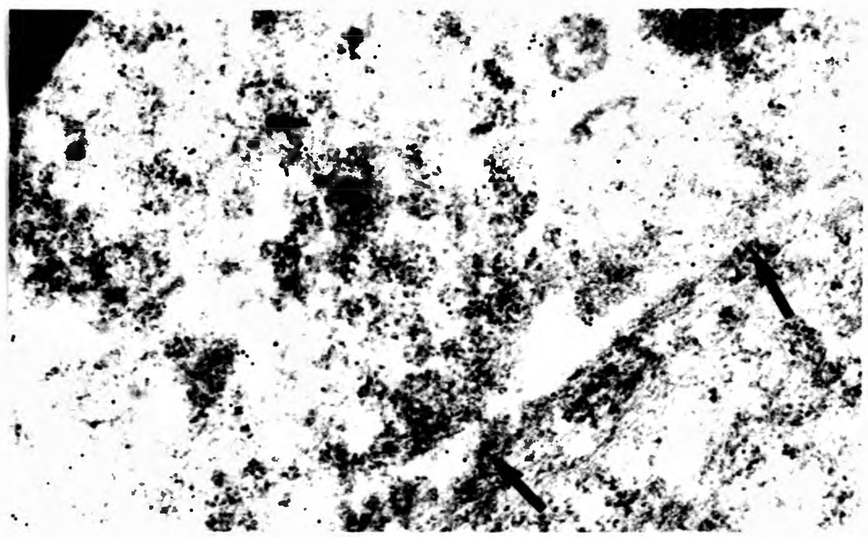
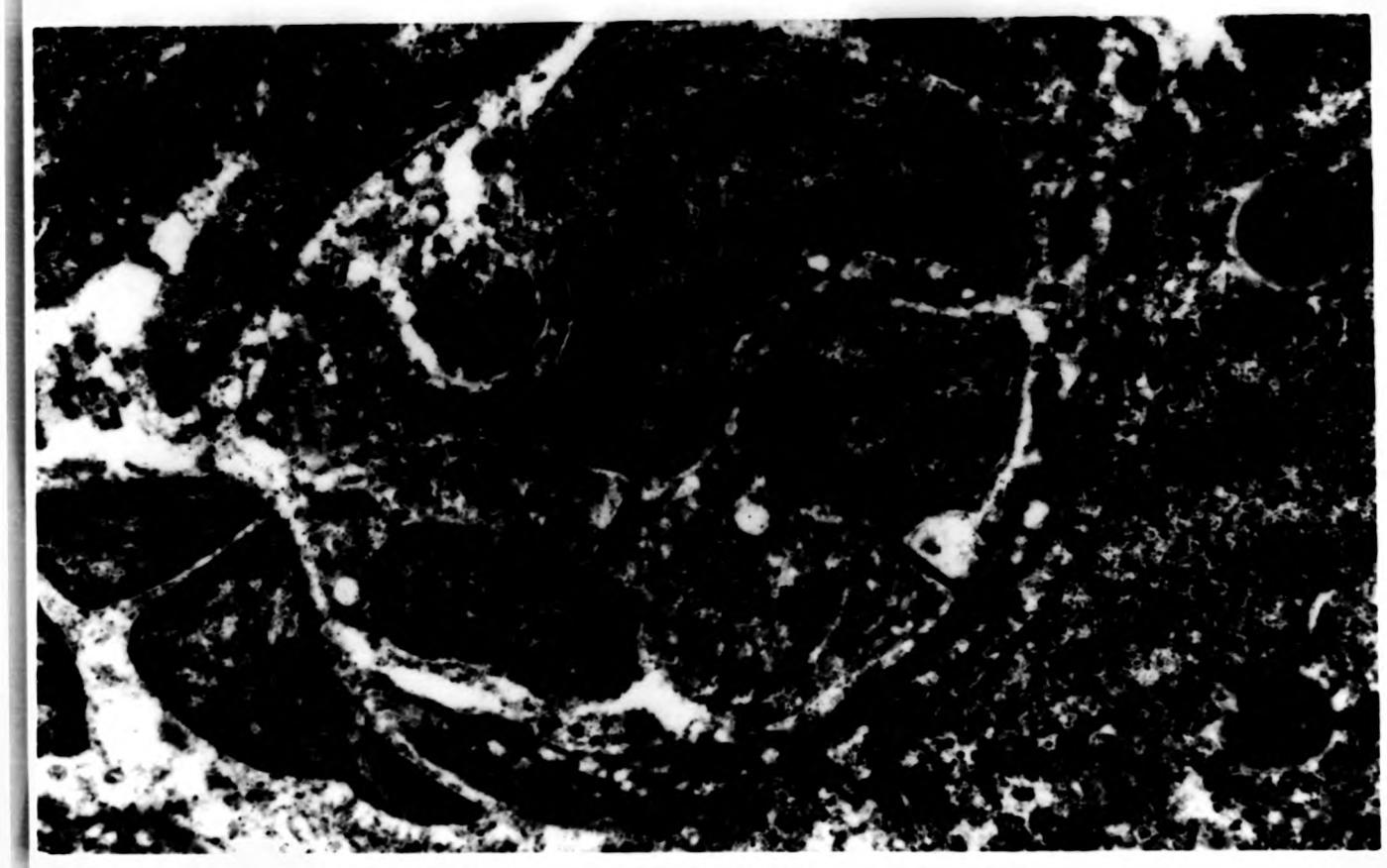
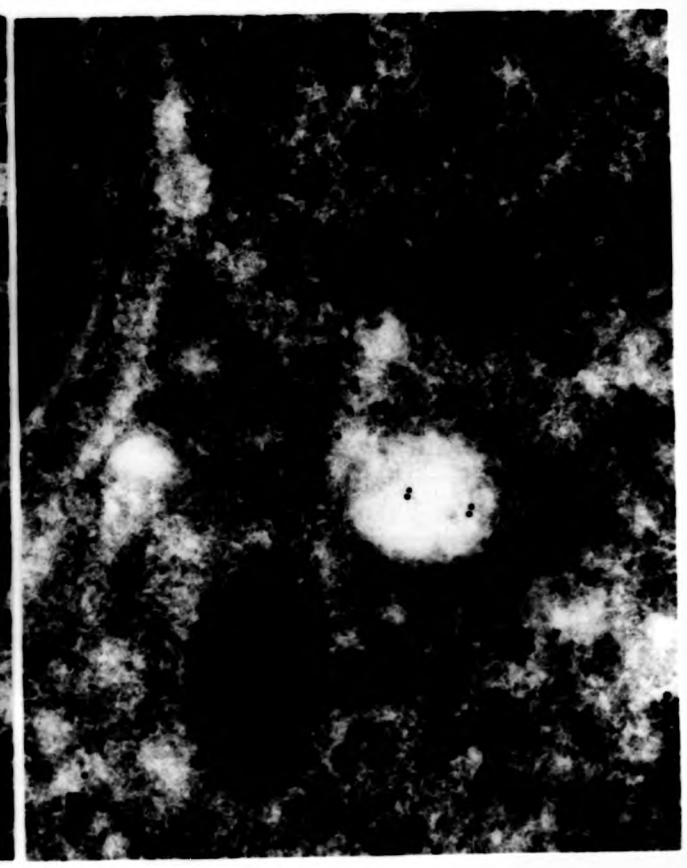


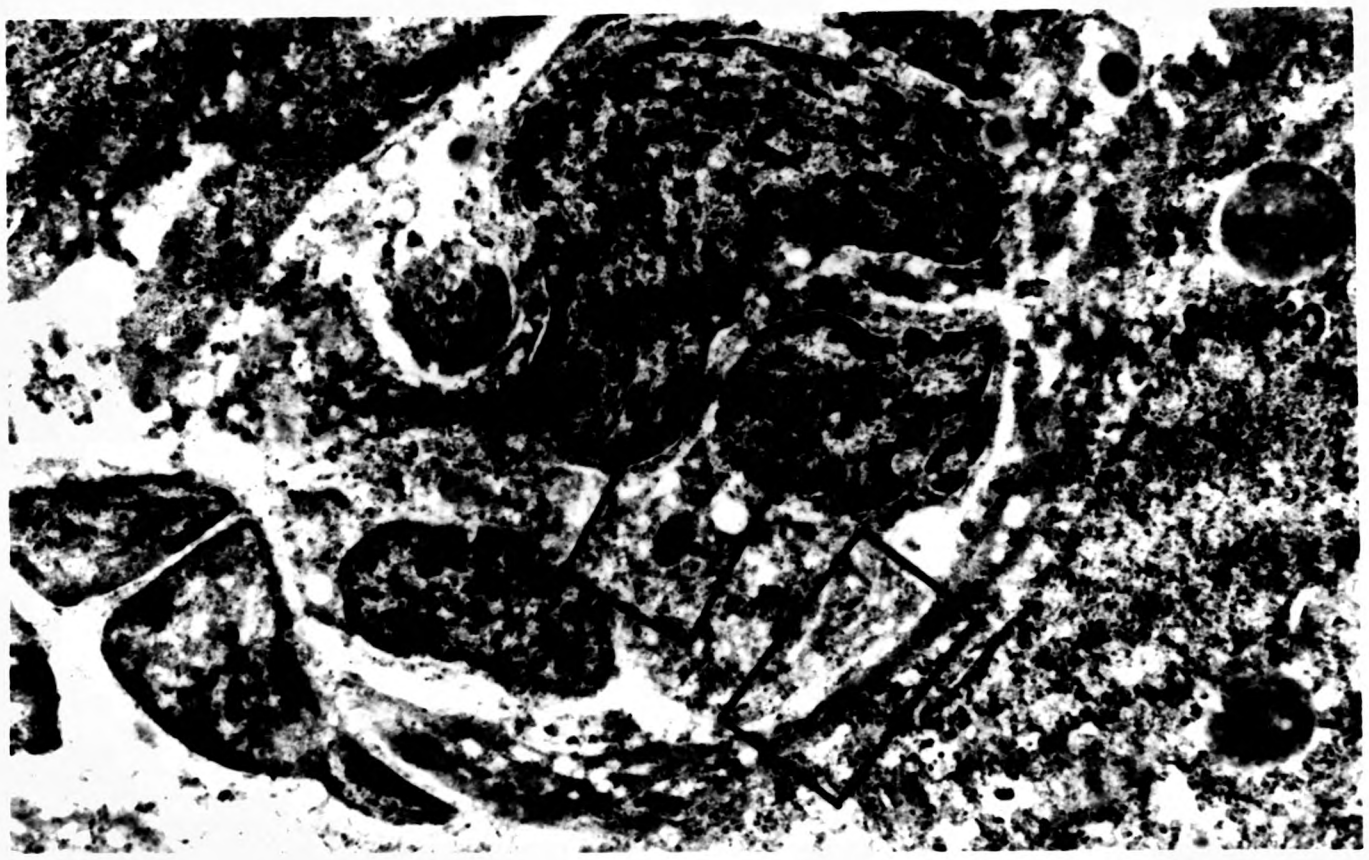
FIGURE 86 - Magnification of the limited area in figure 85. Notice the gold particles which are much more numerous on the cytoplasm of the lymphocyte-like cell. The separation between both cells is ill defined (arrows). x 47 400.

FIGURE 87 - Electron micrograph of a mature female epidermis, fixed with Karnovsky, embedded in Lowicryl and treated with rabbit serum anti-trout IgM and GAR - 10. An apparently multinucleated cell is shown with intense labelling over the cytoplasm. The cell is surrounded by filament-containing cells. Magnifications of the limited areas in the micrograph are shown in figure 88. x 11 400.

FIGURE 88 - A and B - Magnifications of the limited areas on figure 87 showing the gold particle distribution. The poor preservation does not provide a good definition of the ultrastructure, and no obvious intracytoplasmic details can be seen. Nuclear chromatin (n). A- x 47 400. B- x 49 700.







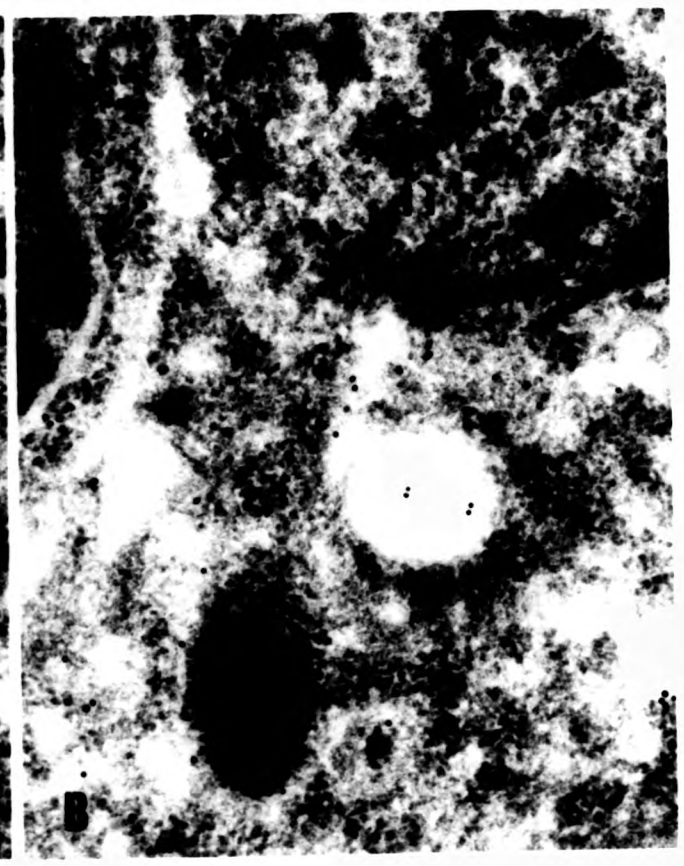
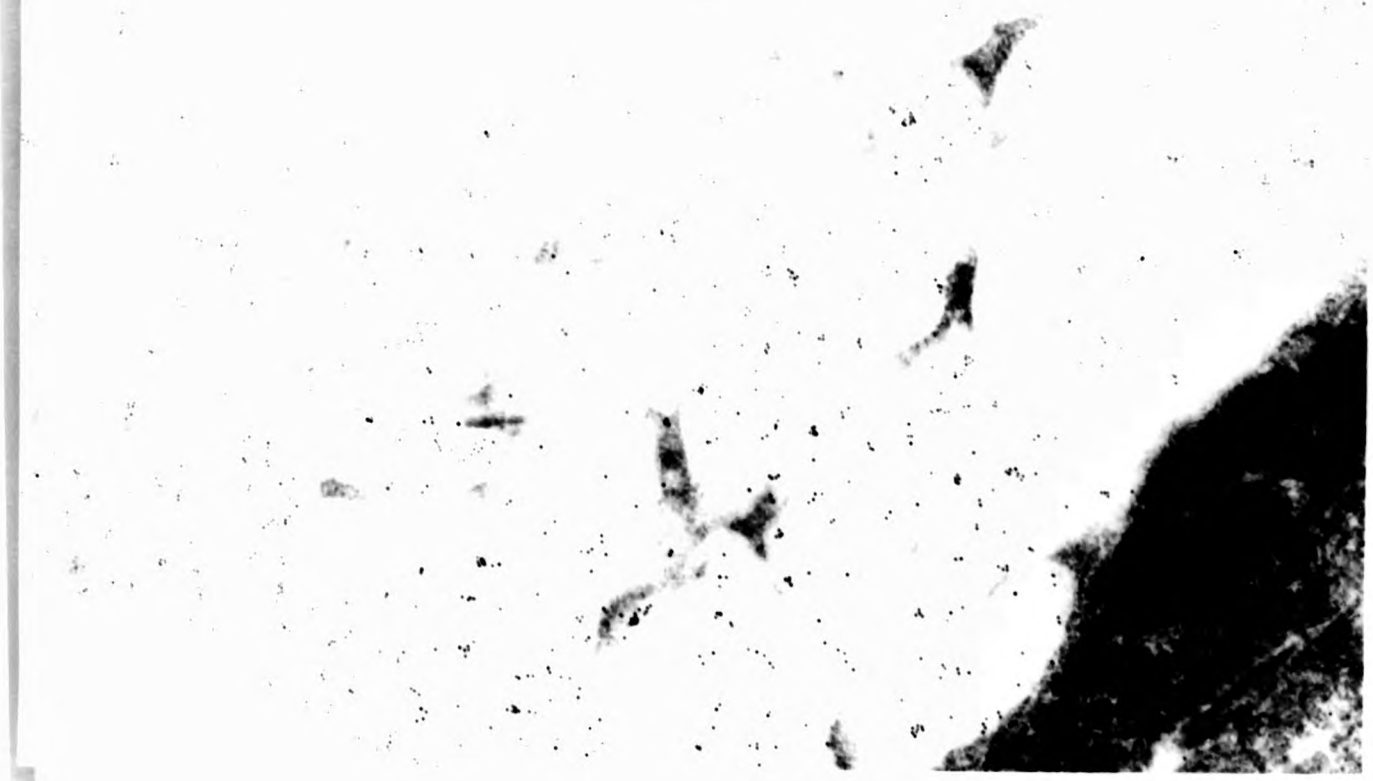
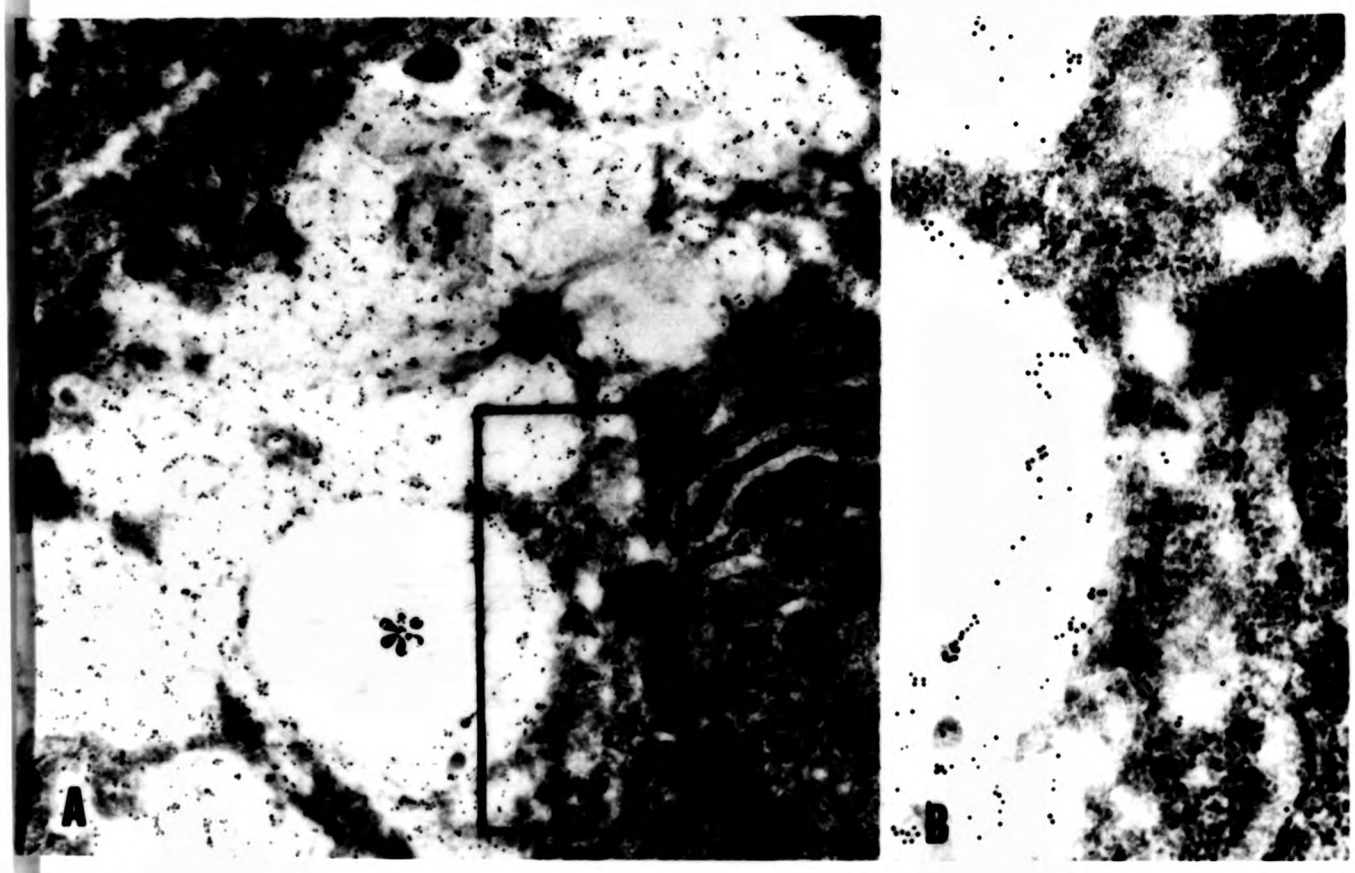
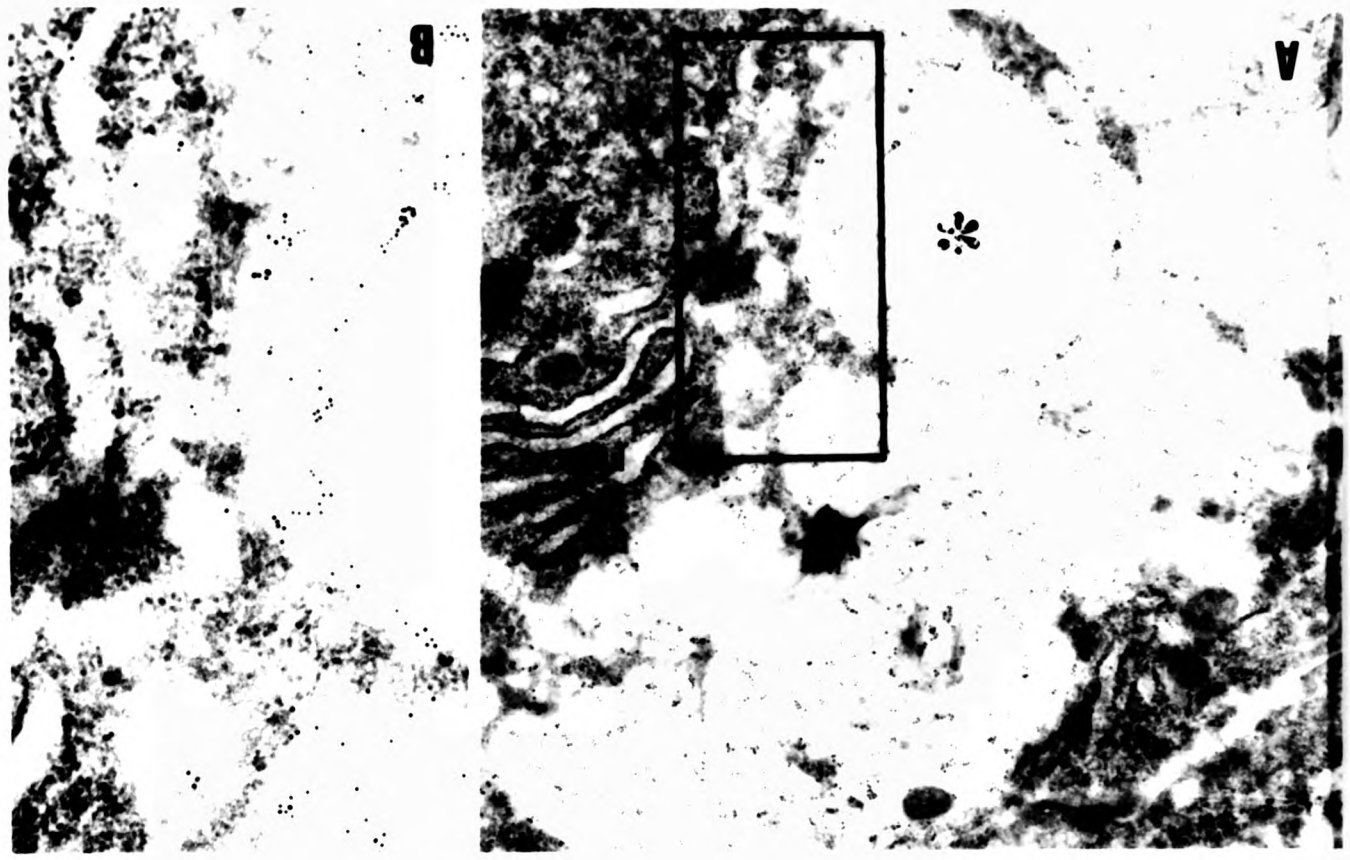

B

FIGURE 89 - Electron micrograph of a mature female epidermis, fixed with Karnovsky, embedded in Lowicryl and treated with rabbit serum anti-trout IgM and GAR-10. Note the intense labelling of the mucous vesicles. No particles can be noticed on the rough endoplasmic reticulum (rer). x 28 700.

FIGURE 90 - A- Electron micrograph of a mature female epidermis, fixed with Karnovsky, embedded in Lowicryl and treated with rabbit serum anti-trout IgM and GAR-10. Notice the intense labelling of the mucous vesicles and the lack of label over the rough endoplasmic reticulum (rer). x 19 300. B . Magnification of the limited area in A. The lack of labelling in the area marked (*) may result from loss of material during the tissue processing. x 38 800.




A
B


B
A

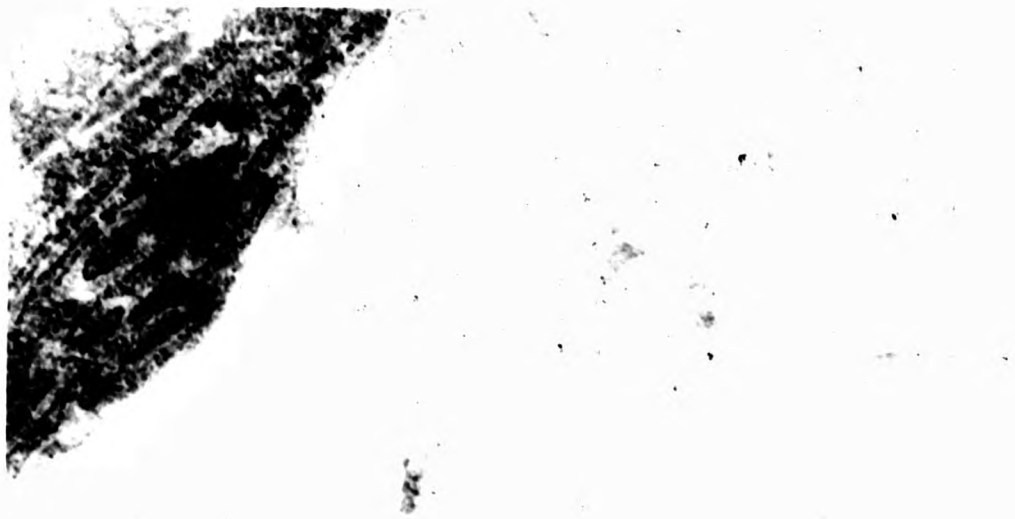
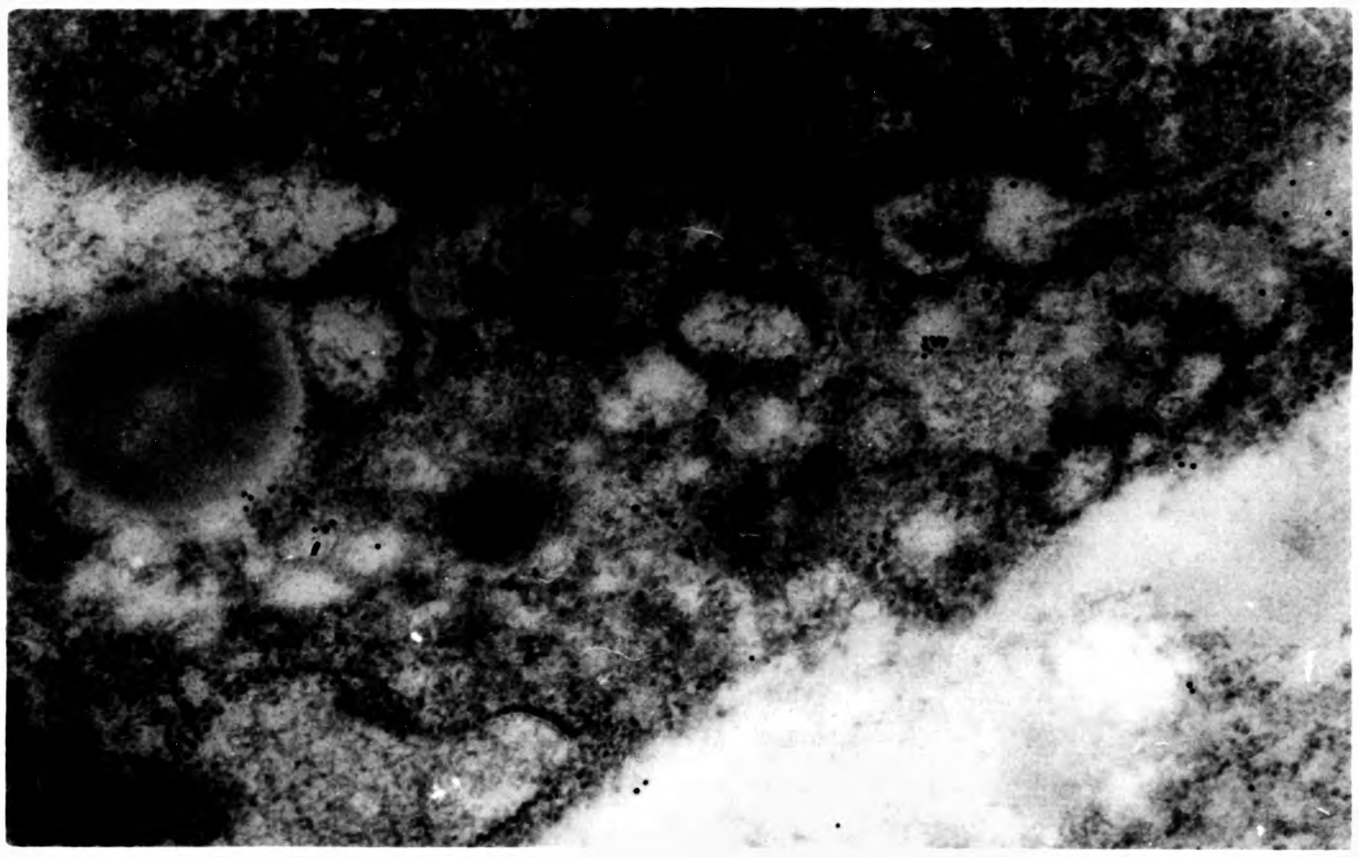
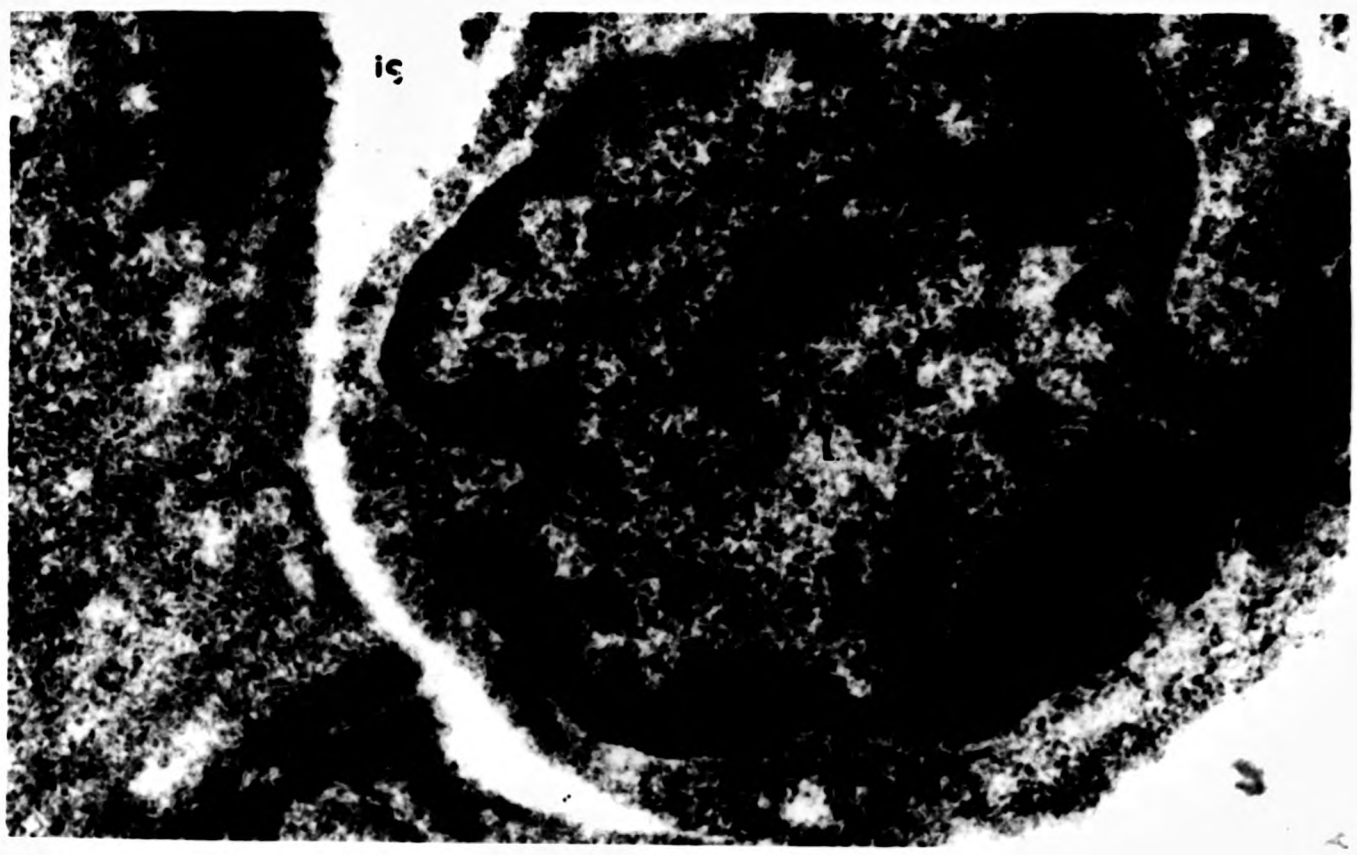
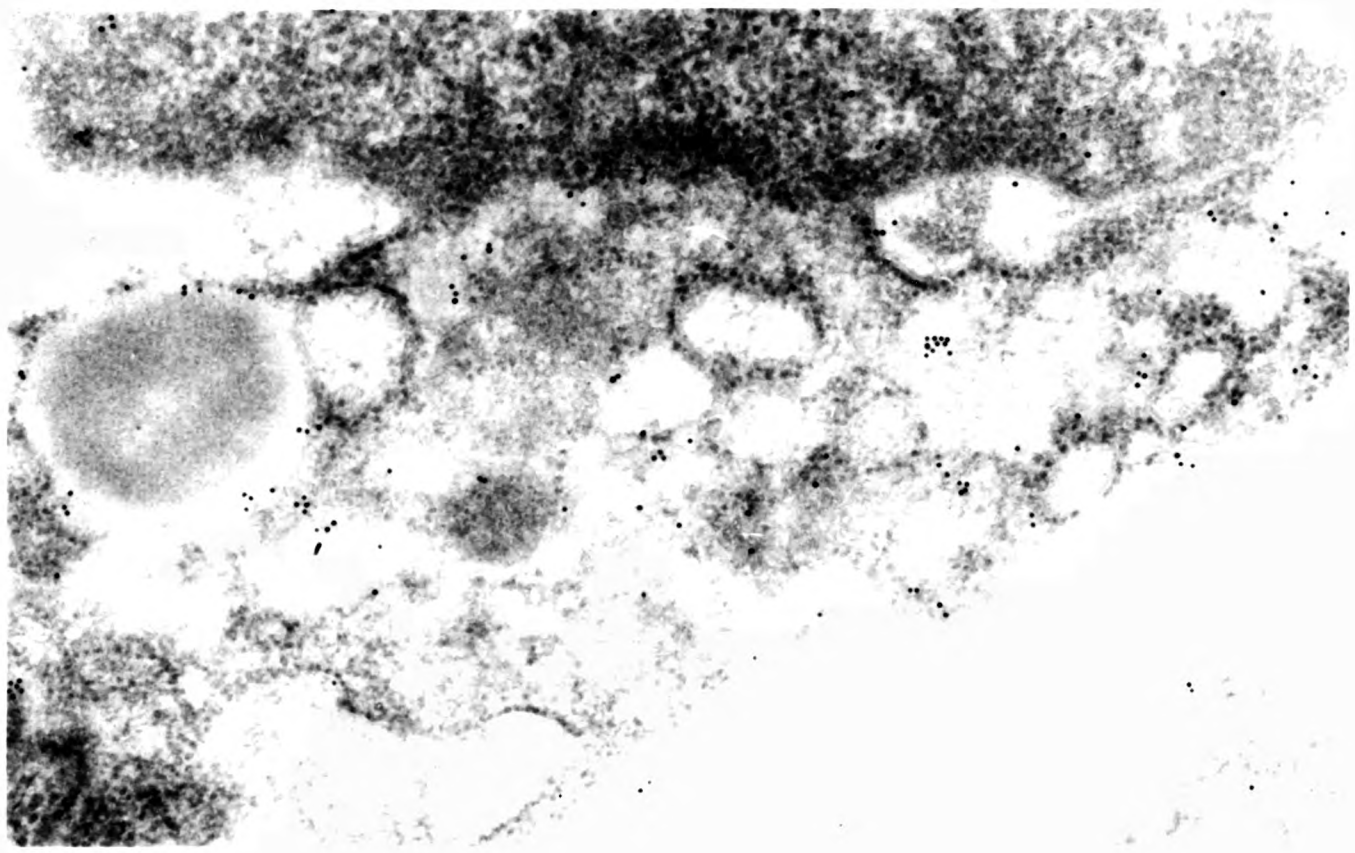


FIGURE 91 - Electron micrograph of mature female dermis, fixed with Karnovsky, embedded in Lowicryl and treated with rabbit serum anti-trout IgM and GAR-10. Notice the gold particles dispersed throughout the cytoplasm of what was interpreted as a macrophage-like cell. x 38 800.

FIGURE 92 - Electron micrograph of mature female epidermis, fixed with Karnovsky, embedded in Lowicryl and treated with normal rabbit serum and GAR-10. Only an extremely reduced number of particles can be seen. Lymphocyte-like cell (L), filament-containing cell (FCC), desmosome (d), intercellular space (is). x 38 800.




is



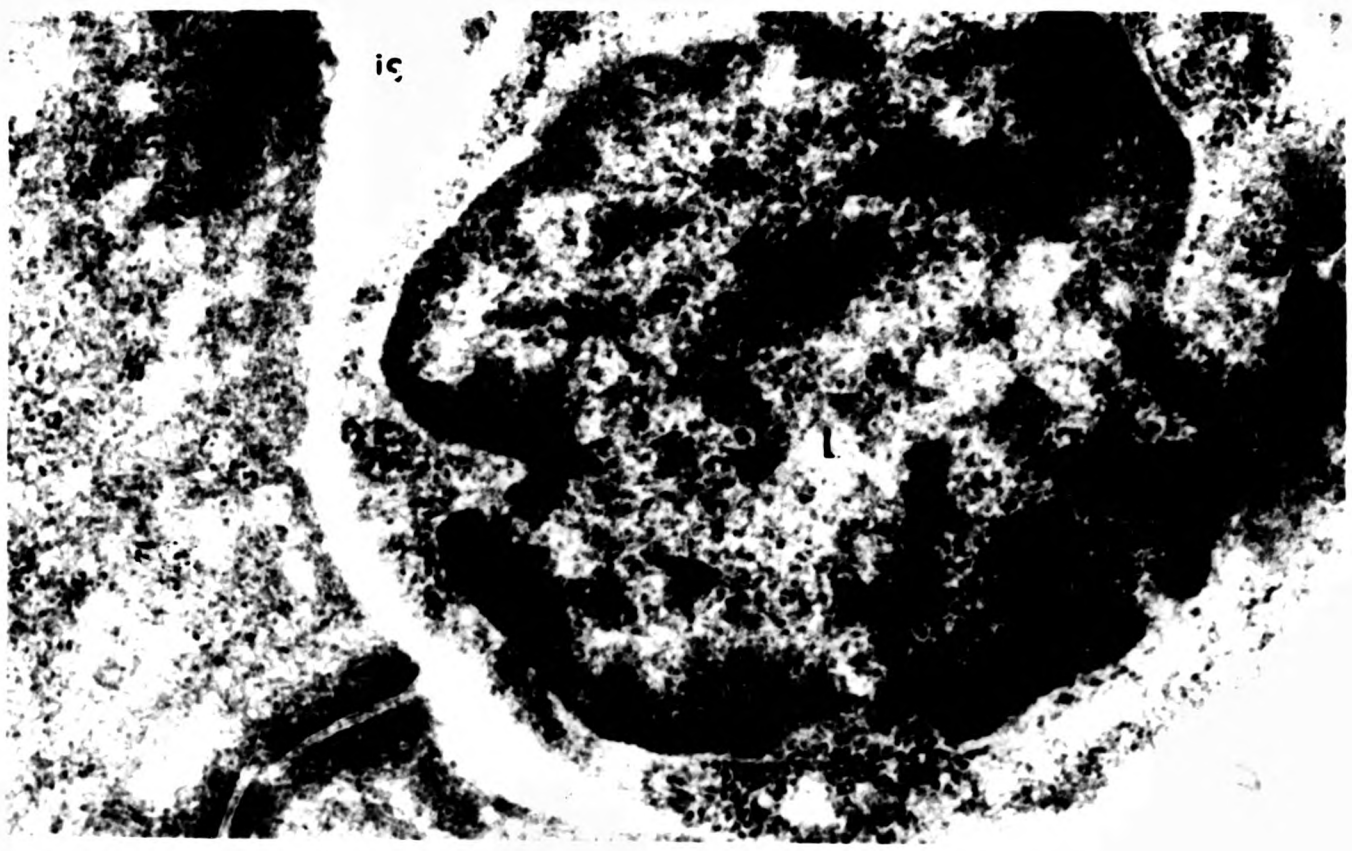

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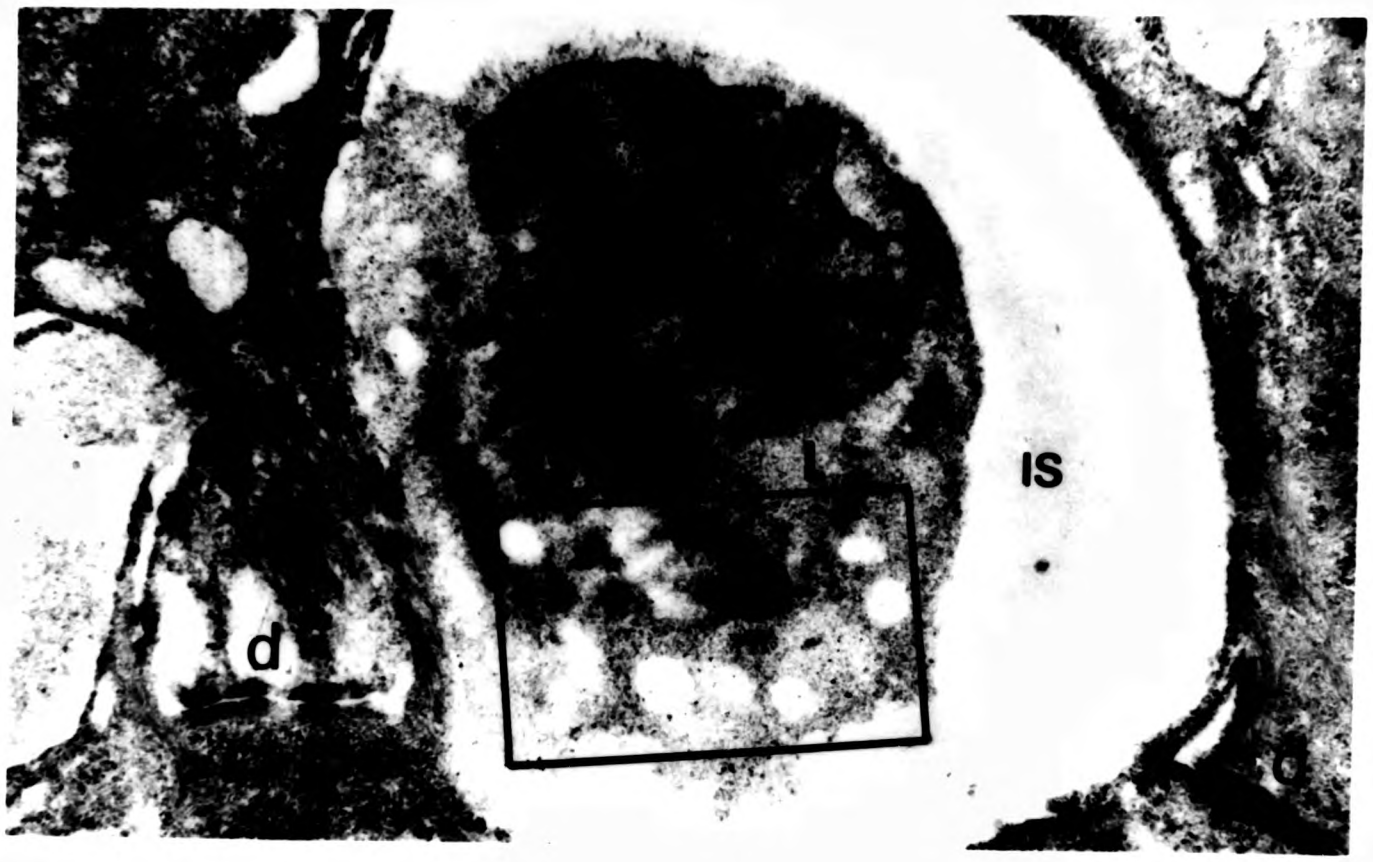
FIGURE 93 - Electron micrograph of a juvenile female epidermis, fixed with paraformaldehyde-picric acid, embedded in Lowicryl and treated with rabbit serum anti-trout IgM and GAR-10. Notice the poor preservation of the material and a labelled lymphocyte-like cell (L). A magnification of the limited area in the micrograph can be seen in figure 94. Nuclei (n), desmosomes between filament-containing cells (d), intercellular space (is). x 20 000.

FIGURE 94 - Magnification of the limited area in figure 95. Notice the apparently random distribution of the gold particles. x 38 800.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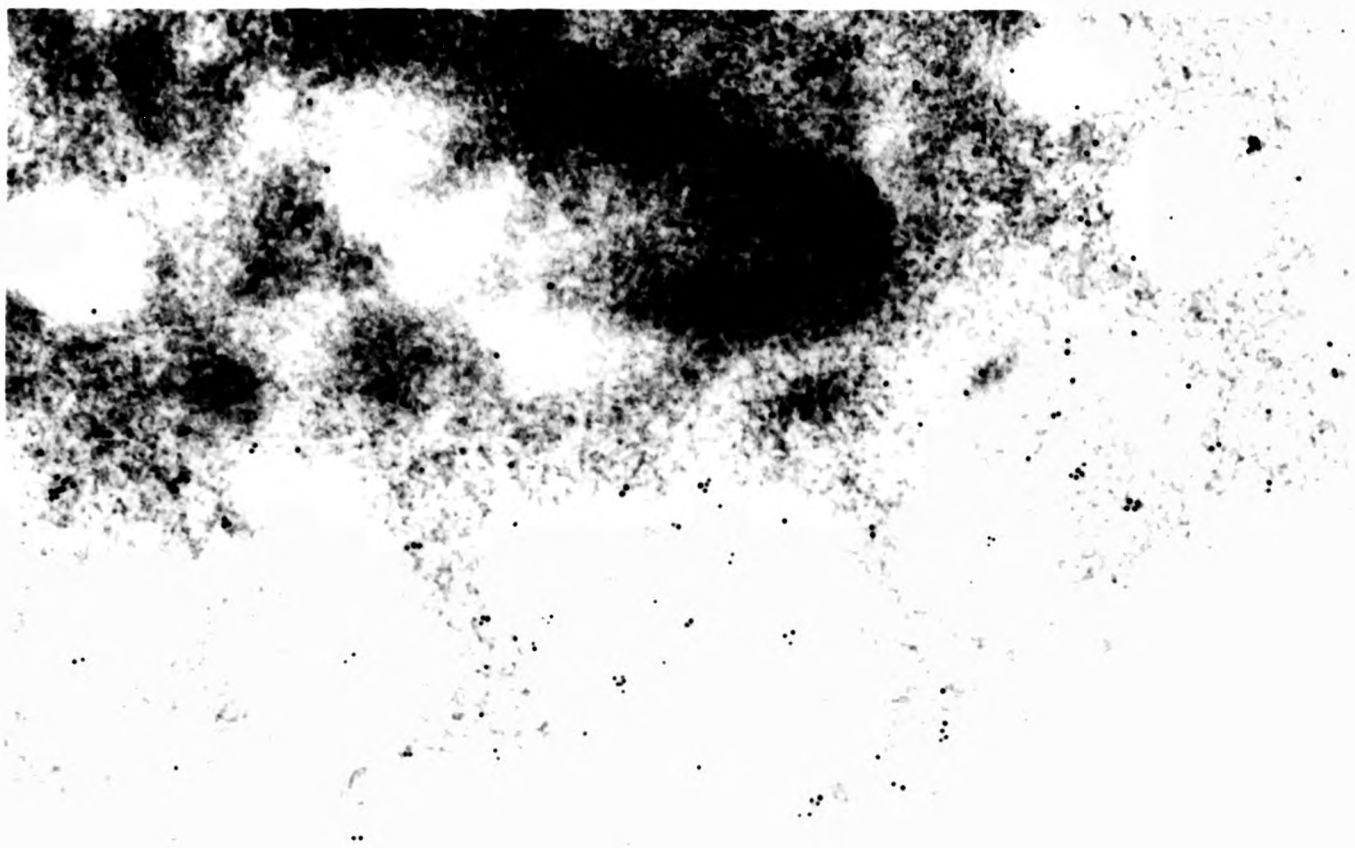

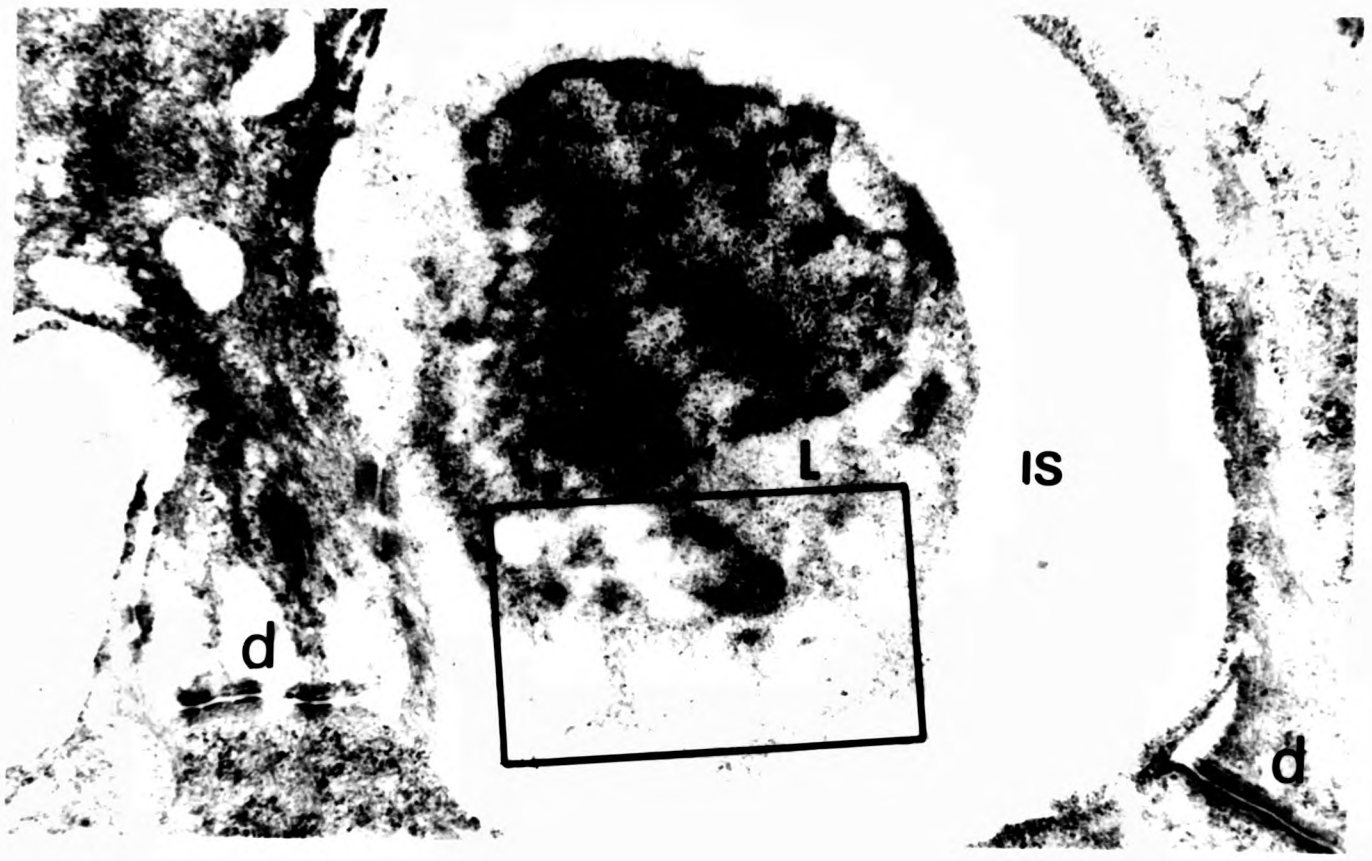
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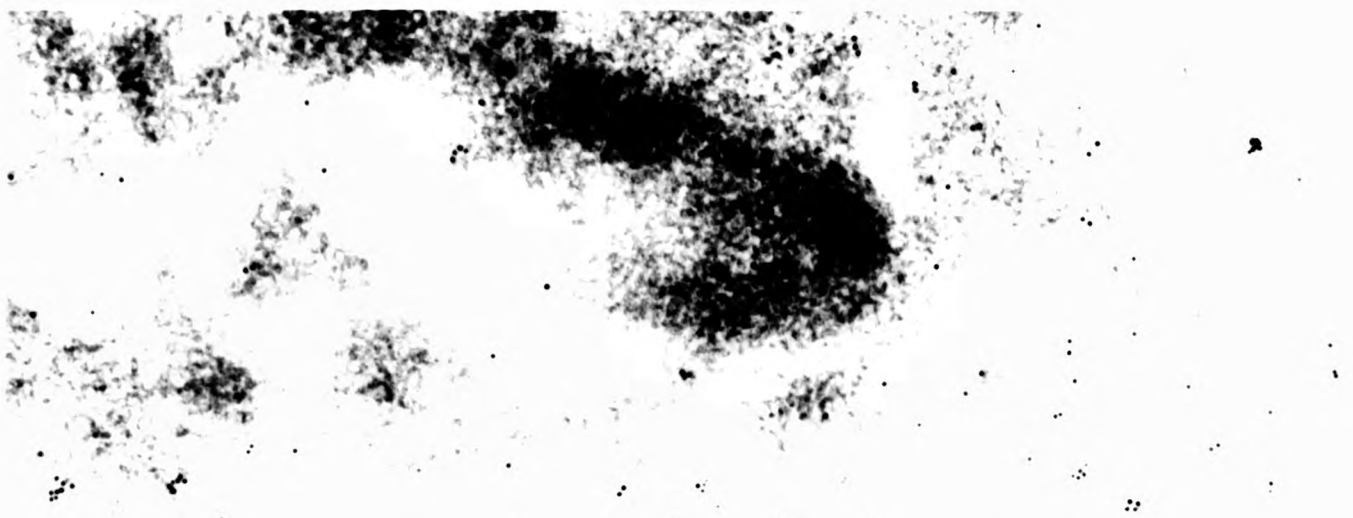
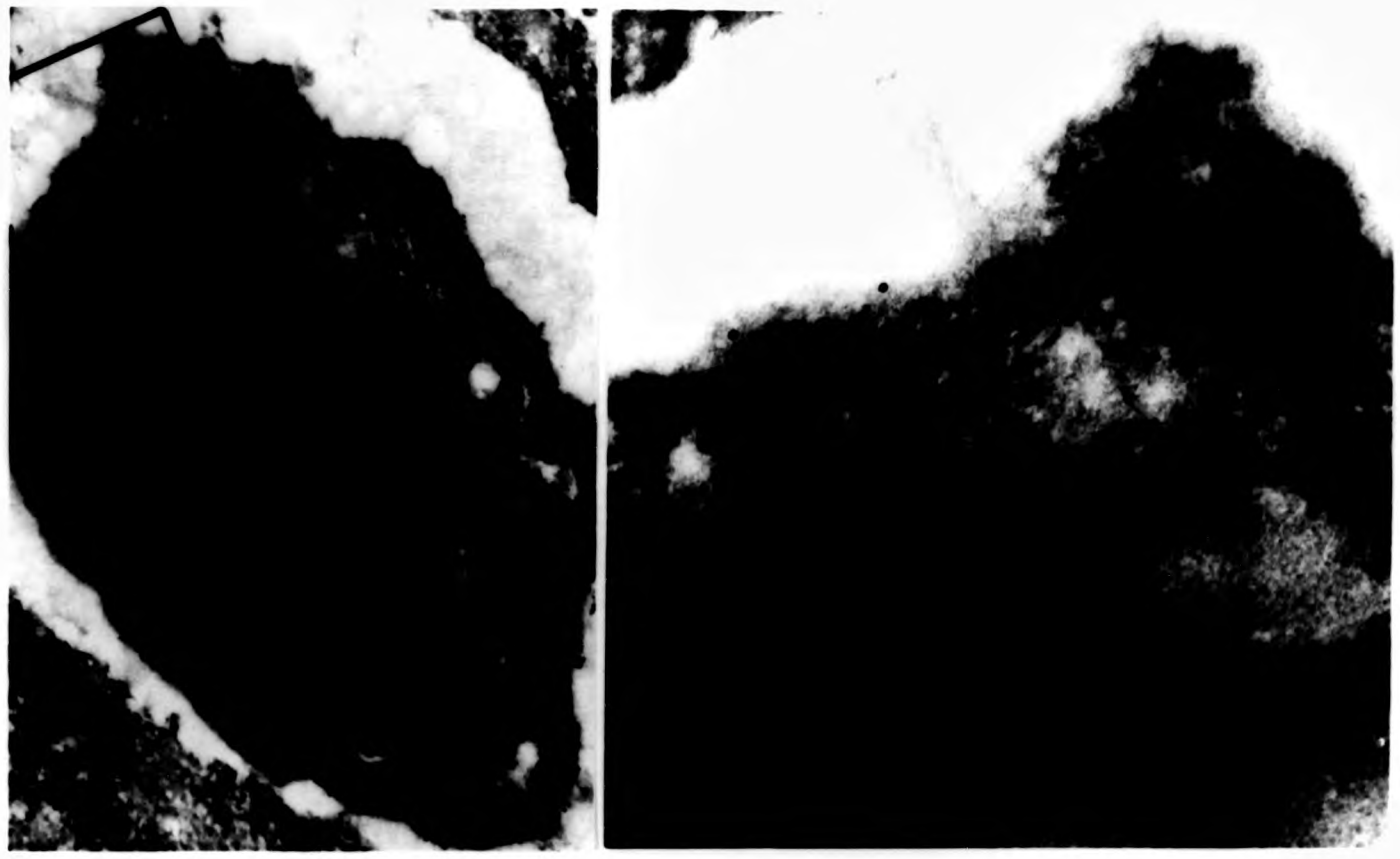
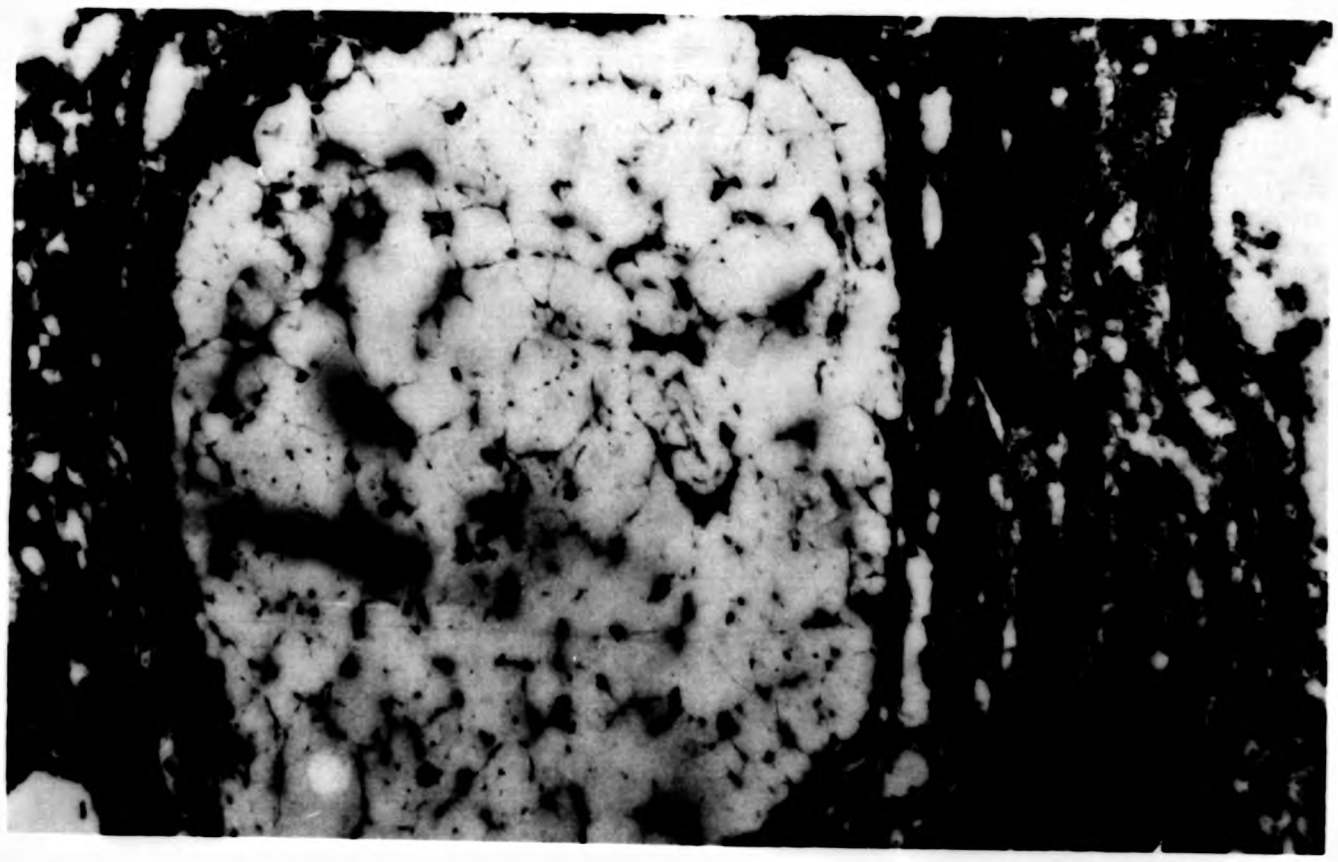
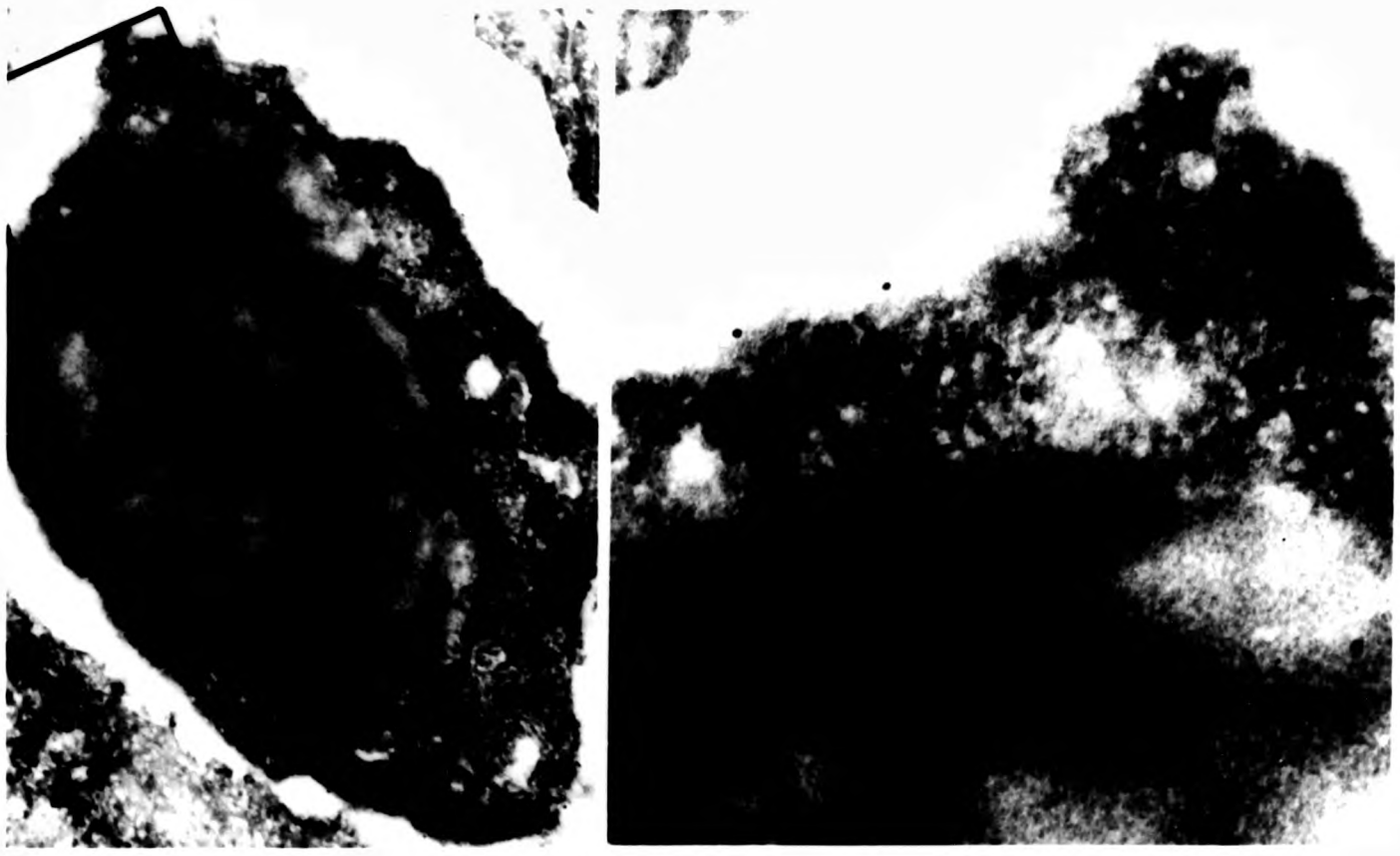


FIGURE 95 - A. Electron micrograph of a juvenile female epidermis, fixed with paraformaldehyde-picric acid, embedded in LR White resin and treated with rabbit serum anti-trout IgM and GAR-10. Notice the lymphocyte-like cell (L) with a few gold particles on the cytoplasm. x 30 000. B. Magnification of the limited area in A. x 60 000.

FIGURE 96 - Electron micrograph of a juvenile female epidermis fixed with paraformaldehyde-picric acid, embedded in LR White resin and treated with rabbit serum anti-trout IgM and GAR-10. No labelling can be seen over the mucous vesicles in the goblet cell. x 12 000.







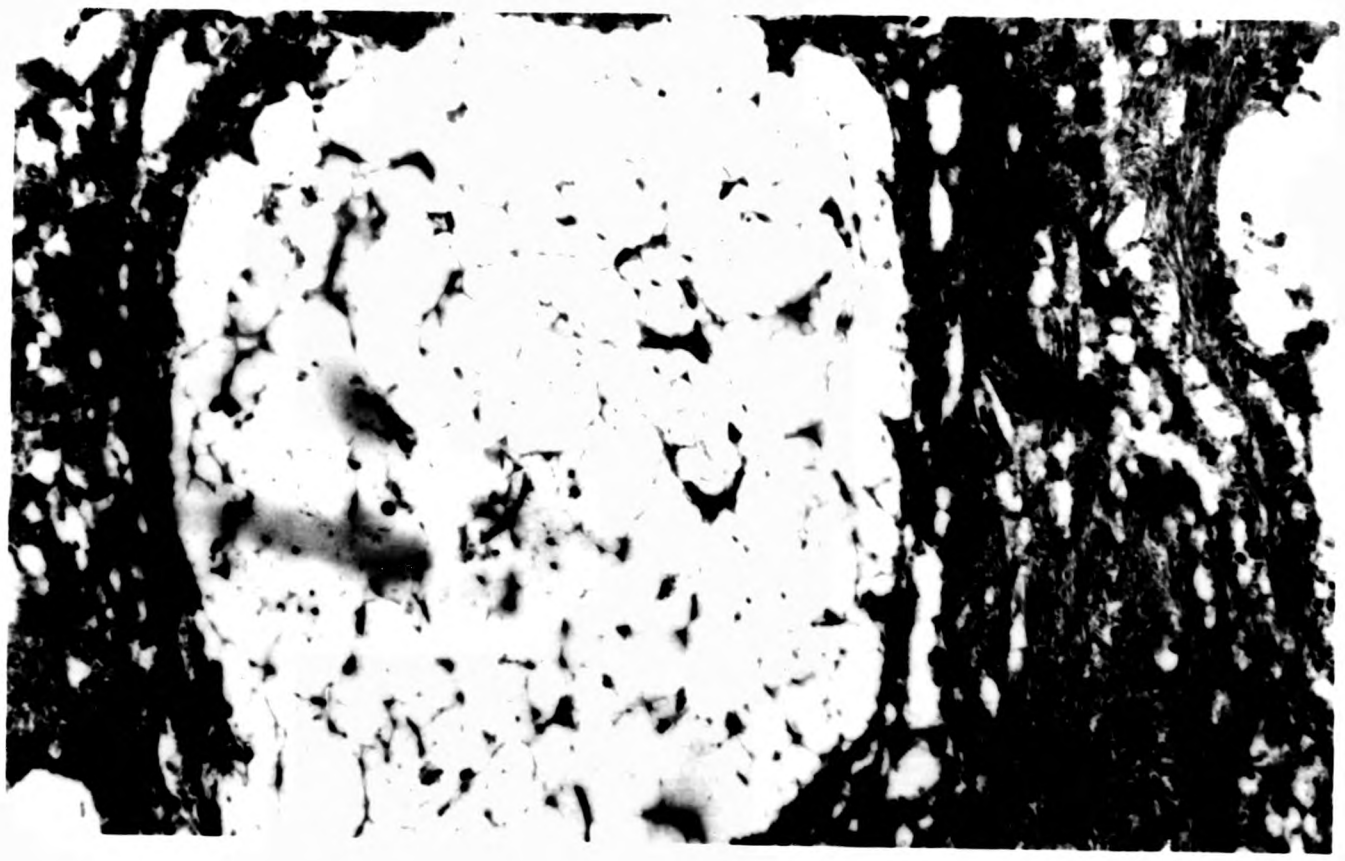


FIGURE 97 - Electron micrograph of juvenile epidermis. This fish was vaccinated by direct immersion in a Vibrio anguillarum bacterin. Mononucleated and multinucleated cells are shown, these last ones presenting a very dense nuclear chromatin. On the upper-left a mucous cell is seen. x 7 600.

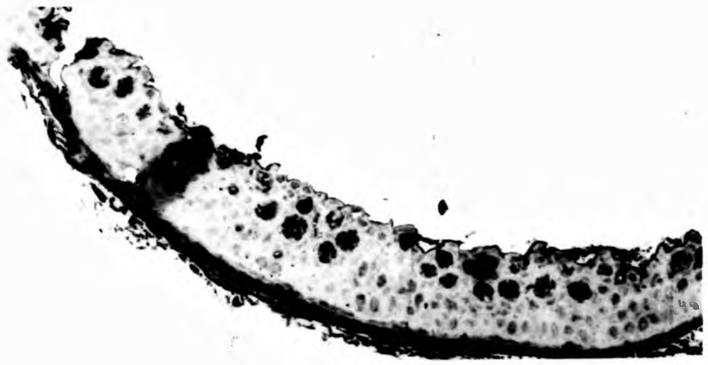


FIGURE 98 - Semithin section of skin of juvenile rainbow trout, vaccinated by direct immersion. The tissue, that was not post-fixed in osmium, was submitted to the immuno-sandwich technique to detect antibodies anti-V anguillarum. Note the positive staining over the mucous cells. x 250.

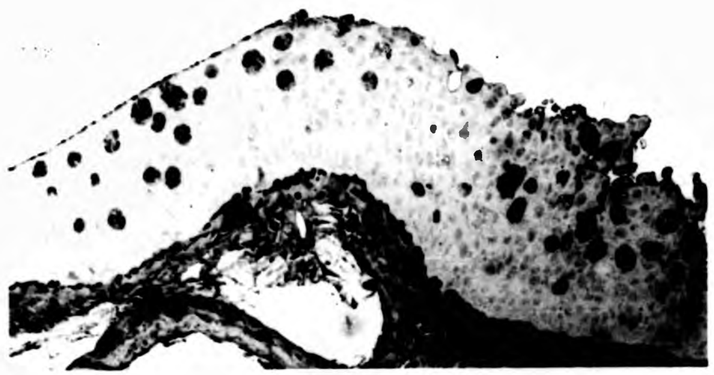


FIGURE 99 - Semithin section of skin of juvenile rainbow trout, submitted to the same procedures as the one indicated in the previous figure. Note the positive staining over the mucous cells. x 250.

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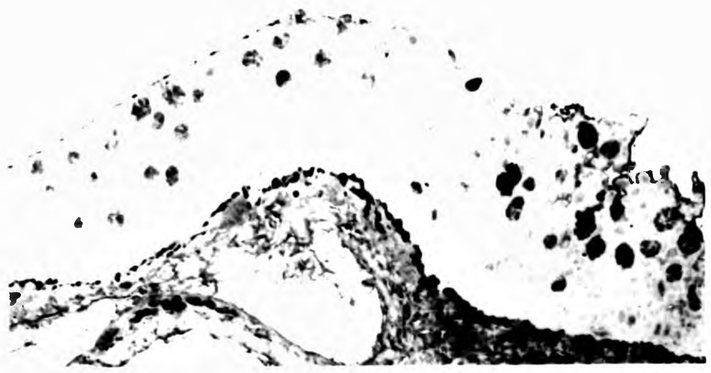

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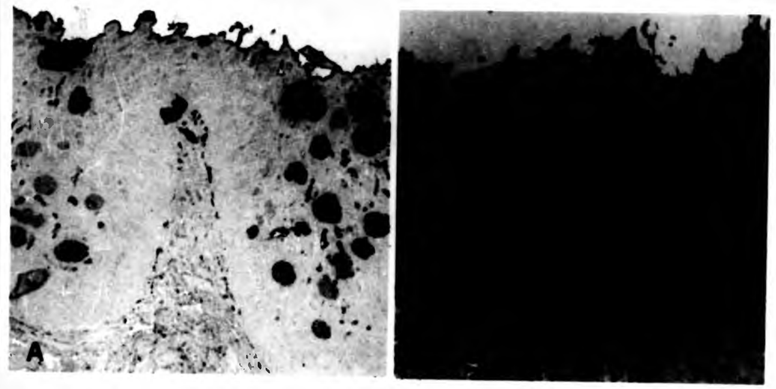


FIGURE 100 - Semithin sections of skin of rainbow trout, non-vaccinated. The tissue was post-fixed in osmium. In A, it was submitted to the immuno-sandwich technique. Note the positive staining over the mucous cells, more intense in some than in others, assuming the appearance of dark granules. In B, control of the technique, in which the rabbit serum anti-V.anguillarum was replaced by normal rabbit serum. x 250.

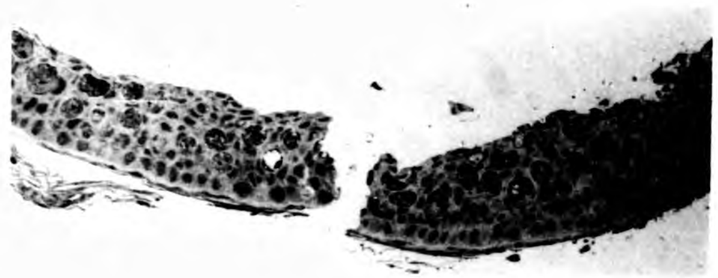


FIGURE 101 - Semithin section of the same tissue shown in figure 98. Control of the immuno-sandwich technique in which the antigen was replaced by tryptic soy broth. x 250.

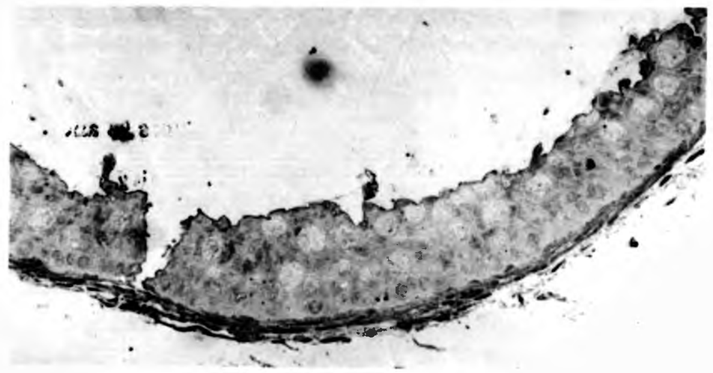
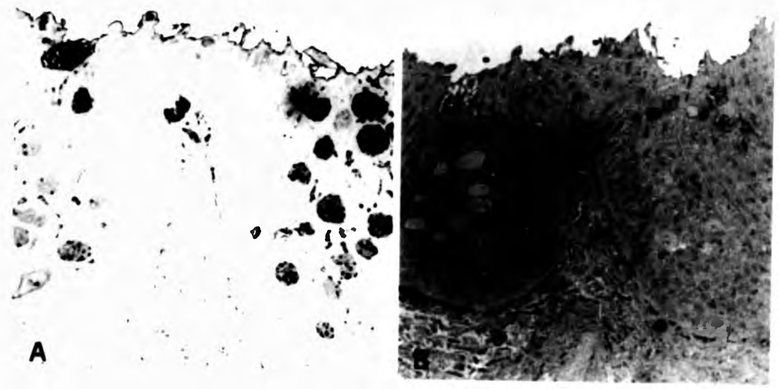


FIGURE 102 - Semithin section of the same tissue shown in figures 98 and 101. Control of the immuno-sandwich technique in which the rabbit serum anti-V. anguillarum was replaced by normal rabbit serum. x 250.



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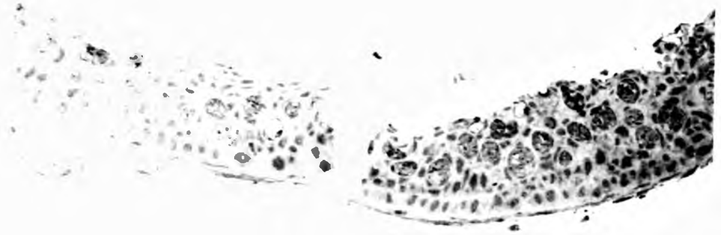

FIGURE 101 - Semithin section of the same tissue shown in figure 98. Control of the immuno-sandwich technique in which the antigen was replaced by tryptic soy broth. x 250.

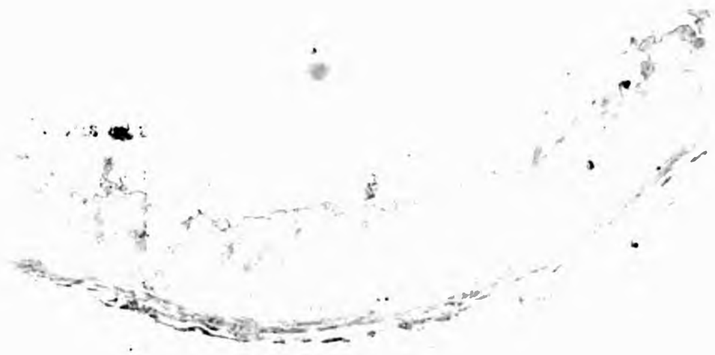

FIGURE 102 - Semithin section of the same tissue shown in figures 98 and 101. Control of the immuno-sandwich technique in which the rabbit serum anti-V. anguillarum was replaced by normal rabbit serum. x 250.