THE STRUCTURE AND GROWTH OF EPIDERMAL LESIONS OF <u>PAROPHRYS</u> <u>VETULUS</u>: A LIGHT AND ELECTRON MICROSCOPIC STUDY

by

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B.Sc., University of Illinois, 1973

### A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

### THE REQUIREMENTS FOR THE DEGREE OF

### MASTER OF SCIENCE

in the Department

of

### Zoology

We accept this thesis as conforming to the required standard

## THE UNIVERSITY OF BRITISH COLUMBIA

September, 1975

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#### ABSTRACT

Natural history, light microscopic, and electron microscopic studies were made of epidermal lesions afflicting juvenile lemon sole (<u>Parophrys vetulus</u>) in the Strait of Georgia, near Vancouver, British Columbia, Canada. Emphasis was placed on the presentation of natural history and histological evidence for the progressive growth of these lesions from an initial morphological form (the angioepithelial nodule) to a mature and morphologically different form (the epidermal papilloma-like lesion). The study of the histological and cytological aspects of the growth of the lesions necessarily incorporated clarification of the nature of unidentified epidermal and stromal cell types which compose much of the mass of the tumour.

It was noted that the histological appearance of the flatfish lesions differs significantly from that of any known fish epidermal disease, including those characterized by cellular hypertrophy and cellular hyperplasia or neoplasia. Natural history studies showed that angioepithelial nodules occurred predominantly on younger fish while epidermal papilloma-like lesions were usually found on larger fish, indicating a progression from the former to the latter. This assumption was supported by the presence of morphological and histological intermediates. The growth of the lesions was characterized by the gradual transformation of normal epidermal cells into "X-cells": ovoid, hypertrophied cells with enlarged nuclei, prominent nucleoli, and necrotic

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cytoplasm. These "X-cell" types completely dominate the mature lesion and are enclosed, or supported, by abnormal epidermal cells termed "enveloping cells". It was found that the pattern of subcellular necrosis in flatfish tumour cells differs from necrosis caused by non-specific lethal injuries to cells. This observation suggests that the subcellular changes observed may be a result of cellular transformation rather than a non-specific lethal injury to the cells.

Other natural history studies indicated a decline in the number of tumours per tumourous fish and a decline in the prevalence of lesions as fish size increases. These results indicate that either the diseased fish are selectively removed from the population or that the tumours necrose and are lost by the host. Both possibilities were discussed. No evidence was found to support the hypothesis that "X-cells" are parasitic protozoans. The presence of virus-like particles in the cytoplasm of the enveloping cells of mature lesions was commonly observed. The relationship between these virus-like particles and the disease process, if any, is unknown.

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# LIST OF ABBREVIATIONS USED IN TEXT

epidermal papilloma-like lesion

AEN angioepithelial nodule

EP

# LIST OF ABBREVIATIONS USED IN FIGURES

A	type A X-cell
AEN	angioepithelial nodule
В	type B X-cell
BC	basal cell
BM	basement membrane
Cap	capillary
Con	connective tissue
cf	cytoplasmic filaments
D	cellular remains (debris)
des	desmosome
EC	epidermal cell
Env	enveloping cell
Ep	epidermis
EP	epidermal papilloma-like lesion
er	endoplasmic reticulum
F	fibroblast
g	glycocalyx
ger	granular endoplasmic reticulum
Gran	granulocyte
Gu	guanidophore
IC	cell of the intermediate layer
L	lymphocyte
M	macrophage
Mel	melanocyte
mit	mitochondria

MC	mucous-secreting cell
Mus	muscle
M£	microfilaments
mt	microtubule
nc	nucleolus
ne	nuclear envelope
nu	nucleus
np	nuclear pore
<b>b</b> w	plasma membrane
RBC	red blood cell
rib	ribosomes
S	stromal X-cell
SC	superficial cell
Sc	scale
Sin	sinusoid
SMC	smooth muscle cell
Str	stroma
Trans	transition form between AEN and EP
vac	vacuole
VLP	virus-like particle
х	X-cell, type A or B

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#### ACKNOWLEDGEMENTS

I gratefully wish to acknowledge the cooperation of:

Dr. A. B. Acton, my supervisor, for his patience and critical revision of this thesis;

Drs. J. R. Adams and W. S. Hoar, for their critical readings of the thesis, and Mr. L. L. Veto, for his excellent and invaluable technical knowledge.

#### INTRODUCTION

### General Introduction

Since 1922 (Harold and Innes, 1922), epidermal skin tumors of unknown aetiology, diagnosed either as epidermal papillomas (Wellings et al., 1964) or as hyperplastic epidermal disease (Nigrelli et al., 1965), have been reported in the lemon sole and other species of the order Pleuronectiformes (families Bothidae and Pleuronectidae), especially along the Paific coast of North America. Several reports indicate relatively high incidences of the disease, often in a particular species and geographic location. It has been noted that tumor incidences in some flatfish are at their highest in the first year of life; no tumors have been reported in flatfish over four years of age (see Mawdesley-Thomas, 1972). Pacis (1932) and Good (1940) reported disease incidences of approximately 5% in lemon sole from Puget Sound, Washington. Ketchen (1953) described similar lesions in a sand sole (Psettichthys melanostictus) population from Hecate Strait, British Columbia. Incidences of 6.4% were reported in the flathead sole (Hippoglossoides elassodon) from the San Juan Islands, Washington; lemon sole, sand sole, and rex sole (Glyptocephalus zachirus) were also afflicted in this area (Wellings et al., 1964; Chuinard et al., 1964; Wellings et al., 1965). Young (1964), in a study of marine life in the San Francisco area, found epidermal lesions on the dover sole (Microstomus pacificus).

Sand sole and rock sole (Lepidopsetta bilineata) from Hecate Strait were reported to be afflicted with papilloma-like lesions; the authors considered these lesions to be a hyperplastic epidermal disease (Nigrelli et al., 1965). Levings (1967) reported that juvenile rock sole in the Gulf of Alaska and Bristol Bay were affected by tumor-like skin growth with a 10% incidence. Studies in San Francisco Bay by Cooper and Keller (1969) indicate that lemon sole there are afflicted with the disease with incidences of 3 - 28%, depending on the time of the year and geographic location. Two additional species, the starry flounder (Platichthys stellatus) and the butter sole (Isopsetta isolepis), were found to be diseased in Puget Sound (McArn et al., 1968; McArn and Wellings, 1971). Miller and Wellings (1971) described the natural history aspects of the disease in the flathead sole, including tumorigenesis, tumor incidences, and growth and survival rates. Recent Puget Sound studies revealed that up to 9% of juvenile lemon sole sampled were afflicted with the disease (Angell and Miller, 1974). A report of a southern California study of dover sole indicated tumor frequencies of 9% (Mearns and Sherwood, 1974).

The epidermal tumors of flatfish have been histologically described, to some extent, for most species. Two histologically and morphologically distinct tumor types were reported in the flathead sole (Wellings <u>et al.</u>, 1964). These were termed the angioepithelial nodule ("AEN", small,

hemorrhagic nodules) and the epidermal papilloma<sup>1</sup> ("EP", larger, more diffuse, granular lesions ranging up to 60 mm in diameter).

Morphologic transitions between the AEN and the EP were also found, suggesting a progression from an initial AEN to the mature EP (Wellings et al., 1964). Wellings and Chuinard (1964) reported progressive growth of the tumor in the laboratory. A third and uncommon morphologic type, termed the angioepithelial polyp ("AEP"), was described in the sand sole (Wellings et al., 1964); the authors also demonstrated the histological similarities of the lemon sole, rex sole, and flathead sole tumors. Histological descriptions of sand sole lesions (Nigrelli et al., 1964) showed the mature papilloma-like lesions to be identical to the others described. The author also described necrotic and regressive tendencies within the tumor. McArn and his colleagues (1968) and McArn and Wellings (1971) reported that the gross morphology and histology of the lemon sole, starry flounder, and flathead sole lesions are identical. The definite progression of the disease from one morphological type (AEN) to another (EP) was

l(NOTE: "Papilloma", in a strict sense, refers to a benign neoplasm. Since, in this case, the true neoplastic nature of the lesion is doubtful (Nigrelli <u>et al.</u>, 1965; Wellings, 1969A; Mawdesley-Thomas, 1972), a more correct term would be "papilloma-like lesion" (see Willis, 1967). For the sake of clarity and continuity, the term "epidermal papilloma" will be used in this paper to describe the mature lesion. Also, the term "tumor", in this report, is used freely in referring to any hyperplastic or neoplastic growth.)

postulated on the presence of morphological transitional stages, the correlation between tumor type and size of fish (smaller flatfish had mostly AENs; larger fish had mainly EPs), and the laboratory observations of disease progression (McArn et al., 1968). It should be noted that other fish exhibiting epidermal papillomas also show the same series of events: the initial appearance of an angioepithelial-type lesion which progresses into a papilloma (Lucke, 1938; also see Wellings, 1969B). Several Japanese reports, although rather unclear, seem to indicate that two species of flounder (Kimura et al., 1967; Honma and Kon, 1968) and several gobiid fishes (Oota, 1952; Imai and Fujiwara, 1959) may be afflicted with histologically similar papilloma-like tumors. Because the epidermal lesions of the various species described show identical structure and morphology, it is generally concluded that the underlying mechanisms of the disease are similar, if not identical. Although the progression from AENs to EPs is strongly suggested by available evidence, no histological progression studies have been done to date.

Histologically the flatfish tumors are unique, as shown by initial light microscopic studies (Wellings <u>et al.</u>, 1964; Nigrelli <u>et al.</u>, 1965; Wellings <u>et al.</u>, 1965; McArn <u>et</u> <u>al.</u>, 1968; McArn and Wellings, 1971). Electron microscopic studies showed marked morphological differences between normal epithelial cells and epithelial cells found in flathead sole epidermal papillomas (Wellings <u>et al.</u>, 1967; Brooks <u>et al.</u>, 1969). Unidentified cell types, termed "X-cells"

(Brooks <u>et al.</u>, 1969) were described from the stromal and epidermal components of the lesions. The nature of these cells was not determined; the authors postulated the cells to be either parasitic protozoans or transformed fish cells.

Comparisons between epithelial tumors of flatfish and other epidermal diseases known in fishes are inevitable. Such comparisons may be useful, especially when using histological structure as a diagnostic basis. The structure of the flatfish tumors is unlike histologic descriptions of cellular hypertrophic diseases of fish epidermis, such as epitheliocystis disease (Hoffman et al., 1969; Wolke et al., 1970), lymphocystis disease (see reviews by Weissenberg, 1965; and Nigrelli and Ruggieri, 1965), and microsporidian infections (Sprague, 1968; Weissenberg, 1968). These lesions are characterized by excessive cellular hypertrophy, usually accompanied by evidence of the initiating organism. The flatfish tumors also differ from histological descriptions of cellular proliferative lesions of fish epidermis. Proliferative lesions include fish pox (e.g., Mawdesley-Thomas and Bucke, 1967), hyperplastic epidermal diseases (Smith, 1935; Nigrelli, 1948; Walker, 1969a and 1969b), proliferations due to infections (Nigrelli and Smith, 1940), epidermal papillomas (Coates, Cox, and Smith, 1938; Lumann and Mann, 1956; Russell and Kotin, 1957; Deys, 1969; Steeves, 1969; Koops et al., 1970), and epidermoid carcinomas (Lucke and Schlumberger, 1941; Tavolga, 1951; Arnowitz et al., 1951; Stolk, 1953, 1956, 1958, and 1960). These proliferative

reactions may or may not show a papillomatous growth structure. The hyperplastic or neoplastic cells tend to retain the form and function of the cell type of origin.

Generally, the cells tend to retain their normal staining ability with many stains. Usually no necrotic or cellular degenerative processes are apparent. Most hyperplastic or neoplastic growths tend to exhibit the same histologic pattern described by Lucke and Schlumberger (1949) for epidermal papillomas. It can be noted, by using histological comparisons, that the epidermal tumors found on flatfish differ structurally from other described epidermal lesions of fishes.

Recently it has been noted that this disease is quite common in juvenile lemon sole in the Strait of Georgia, near Vancouver, British Columbia. Similar lesions were also found in small numbers or isolated cases of several other species (sand sole, rock sole, speckled sanddab, rex sole, starry flounder, and flathead sole). Natural history data, light microscopy, and electron microscopy were utilized in the study of this disease, as manifest in this population of juvenile lemon sole.

Insofar as the nature of a disease must be known before its aetiology and consequences can be meaningfully examined, emphasis was placed on the clarification of two unresolved aspects of the nature of these lesions: 1) the histogenetic relationship between AENs and EPs, and 2) the nature of the unidentified "X-cell" type which dominates the tissue of the mature lesion.

The primary objectives of this thesis are:

 to present natural history and histological evidence for, and descriptions of, the progressive growth of these lesions, and

2) to clarify, histologically and ultrastructurally, the nature of the unidentified "X-cell" type and their relationship to the histological growth and structure of the lesions.

Hopefully this type of study of these epidermal lesions and their unique histology will help to clarify the nature, aetiology, and consequences of the disease.

### Life History Information

The prevalence of any disease occurring in a natural population (especially infectious diseases) generally is intimately associated with the natural history of the species affected. This seems to be the case with flatfish epidermal lesions. For this reason, a short synopsis of the life history of the lemon sole follows.

The lemon sole (<u>Parophrys vetulus</u> Girard, 1854) is a member of the teleost family Pleuronectidae (Pleuronectiformes; "flatfish"). The natural history of this species has been well documented (see Hart, 1973); however, little is known about the habits of the juvenile fish. In British Columbia, spawning may occur from January to March (Taylor, 1946). Lemon sole larvae, like other flatfish, are symmetrical and pelagic for six to ten weeks before they undergo

metamorphosis and settle to the bottom. In southern British Columbia metamorphosing larvae are found on the beaches in mid-April; when metamorphosis is complete the fish generally measure around 10 mm. Growth is rapid (23 mm per month) in the spring and summer months of the first year of life; they reach 100 mm total length by August. Lemon sole average 125 mm at the end of one year of life; after two years they average 200 mm (Ketchen, 1947; Forrester, 1969). Growth is extremely slow during fall and winter months (Ketchen, 1947; Manzer, 1951; Smith and Nitsos, 1969; van Cleve and elsayed, 1969; Forrester, 1969a); other studies have shown that in mid-winter lemon sole undergo periods of semihibernation and no food intake. There is a definite yearly movement of adult fish into deeper waters in the winter and a return into shallow waters in the spring. Like most flatfish, juvenile lemon sole tend to congregate on certain "nursery grounds" where they remain until mature (Rae, 1965). Studies of lemon sole migratory habits have shown that, as a rule, extensive migrations are not undertaken and populations generally remain distinct (Ketchen and Forrester, 1955; Forrester, 1969b). Lemon sole on the British Columbia coast are characteristically found in various isolated populations at the heads of many inlets (Hart, 1973).

#### MATERIALS AND METHODS

### Collection Procedures and Data Analysis

Normal and tumour-bearing lemon sole were collected regularly from a sampling area near the mouth of the North Arm of the Fraser River, near Vancouver, British Columbia. Other areas in the Strait of Georgia were sampled irregularly.

Flatfish were collected at depths of 1 - 20 fathoms, with either an otter trawl or a door trawl towed at slow speed. All flatfish under 180 mm total length were removed and taken to the laboratory; larger flatfish were released after being examined and recorded. Those flatfish removed to the laboratory were either kept in tanks or killed and preserved in 10% formalin. All fish were examined for evidence of epidermal lesions and separated into normal and tumorbearing groups. All flatfish were individually described by species, total lengths, and weights. In addition, drawings were made of each tumor-bearing flatfish, illustrating the position, relative size, coloration, and general macroscopic description of each lesion.

Epidermal lesions found on flatfish are of two basic morphologic types with a continuum of intermediates. The tumors can be classified morphologically as angioepithelial nodules (AENs), epidermal papillomas (EPs), and AEN/EP transition forms, as previously described (Wellings <u>et al</u>., 1964). These classifications will be used in descriptions

of tumors studied histologically; each type has distinctive morphologic and histologic characteristics, as shown in Figure 1 - 6. Tumors classified (morphologically) as AENs were small, hemispherical, white-to-red, smooth-surfaced nodules located anywhere on the body; the diameters of the nodules varied from 1 - 5 mm. Those tumors classified as AEN/EP transition stages showed characteristics of both lesion types. They generally were large (3 - 7 mm in diameter) hemispherical growths with spreading, plaque-like edges, or with a rough, granular surface (indicative of thickening and infolding of the epidermis). Epidermal papilloma type tumors were generally large (covering up to one-half of one side of some fish), raised, granular, gray-to-black lesions with spreading, diffuse edges; many non-diffuse, clearly defined epidermal papillomas were also noted, although in smaller numbers. The sessile papillomas (with broadly based, graded edges) and the pedunculated papillomas (with a narrow base and well-defined edges) showed identical histological structure and for that reason were grouped as one type. Both morphological types of EP lesions exhibit the so-called "cauliflower-like" appearance shown by other epidermal papillomas in other fishes.

The grading of epidermal lesions on the basis of external morphology can be subjective, especially when considering an infinite variety of intermediates between the two basic tumor types. Correctly, any flatfish epidermal lesion with spreading, plaque-like edges or with a granular-

### PLATE 1 Figures 1-6

Fig. 1

External morphology of tumour type designated as angioepithelial nodules (AEN); on juvenile lemon sole. X 3/4.

Fig. 2

External morphology of tumour type designated as AEN/EP transition forms (Trans); on juvenile lemon sole. X 3/4.

Fig. 3

External morphology of tumour type designated as EP-like (EP) lesions; on juvenile lemon sole. X 3/4.

Fig. 4

Typical histological structure of AEN tumour stage. Epidermis appears normal. Hematoxylin/eosin. Approx. X 3.5.

Fig. 5

Typical histological structure of AEN/CP transition forms. Epidermis is slightly thickened and infolded; spreading edges are apparent. Hematoxylin/eosin. Approx. X 3.

### Fig. 6

Typical histological structure of EP tumour stage. The epidermis is highly thickened and infolded. Hematoxylin/ eosin. Approx. X 3.



appearing epidermis should be termed an EP; histologically, lesions at these stages show more similarities to EPs than to AENs (see Miller, 1969; Miller and Wellings, 1971). For this reason, only the AEN and EP classifications were used in compilation of the data comparing relative occurrences of tumour types (in Figure 9).

Tumour natural history data in this report are presented as a function of fish size; the data are presented in terms of 5 mm size groups (total lengths). The relationship between fish size and age was not determined by otolith or scale readings. Interpretation of age and size relationships were based on natural history and growth rate data from previous studies of lemon sole growth (see introduction for references); similar growth patterns can reasonably be assumed in the lemon sole population studied.

The analysis of percentages of tumour-bearing fish per size group was based on 3896 lemon sole under two years of age (under 180 mm total length), the total juvenile lemon sole catch from the North Arm sampling site during the period from September 1973 to February 1975. Other analyses of the natural history of the lesions (i.e., average number of lesions per fish, and relative incidences of AENs and EPs) were based on a sample of 510 tumour-bearing fish collected during the period from September 1973 to June 1974.

### Light Microscopy

For light microscopic studies, normal skin and tumorous tissues were fixed in 10% formalin or Carnoy's solution, embedded in paraffin, and sectioned at 5-15 microns. Sections were stained with hematoxylin and eosin, toluidine blue, or by the Feulgen reaction (counterstained with fast green). Histological studies were based on sections from 134 tumors of various types from 38 lemon sole.

### Transmission Electron Microscopy

For ultrastructural studies, normal skin and tumorous tissues were removed from live fish anesthetized with tricaine methanesulphonate (MS-222). These tissues were cut into 1 mm cubes and fixed for 1-2 hours in 2.5% glutaraldehyde (Sabatini et al., 1963) buffered at pH 7.4 with a phosphate buffer (Karlsson and Schultz, 1965). The specimens were post-fixed in 1% osmium tetroxide for 1 hour, dehydrated in a graded series of methanols, and embedded in Epon (Luft, 1961). The blocks were polymerized at 60°C for 20 hours. Thin sections were cut on a Reichert OmU2 ultramicrotome or on a Sorvall MT-2 ultramicrotome. The sections were stained with uranyl acetate (as in Sjostrand, 1967) and lead citrate (Reynolds, 1963) and observed with a Hitachi HS-7S electron microscope. Thick (1 micron) sections were also cut from Epon-embedded material, stained with toluidine blue, and utilized in light microscopic studies.

## Scanning Electron Microscopy

For studies of the surface structure of normal flatfish epidermal cells, small (1 cm) squares of lemon sole skin were removed, fixed in 10% formalin, dehydrated, coated with a thin layer of gold, and examined with a Cambridge Stereoscan scanning electron microscope.

#### RESULTS

### Natural History Data

During this study, a total of 3896 lemon sole were collected from the North Arm sampling site. All fish were under 180 mm in total length and were tentatively identified as being in the first two years of life. A total of 1164 lemon sole were found to be tumour-bearing. These figures indicate a cumulative tumour prevalence of 29.9% for the period from September 1973 to February 1975. However, the percentages of flatfish bearing lesions varied monthly and showed a yearly cycle. Tumourous fish appeared in August or September, showed a peak prevalence in November (approximately 63%), and showed declining frequencies thereafter to a low in June, July, and August (approximately 5%).

Figure 7 shows the relationship between frequency of tumour-bearing fish and fish length. A drastic increase in tumour prevalence is noted between the 50-54 mm size group and the 75-79 mm group. After this increase (to a maximum percentage incidence of 49.0%), further size increases are directly correlated with steadily decreasing tumour percentages until the zero point is reached in the 160-164 mm group.

The relationship between fish size and the number of tumours found on each fish is shown in Figure 8. The histogram shows that the average number of tumors per fish

### FIGURE 7

The relationship of tumour prevalence and fish size.

Histogram illustrating the relationship between the percentage of tumour-bearing fish and fish length, showing a decrease in tumour prevalence as total length increases. Data based on the analysis of 3896 lemon sole sampled from one area in the Strait of Georgia from September 1973 to February 1975.



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Starting points of 5 mm size groups (fish total lengths)

### FIGURE 8

The relationship of numbers of tumours per fish and fish size.

Histogram illustrating the relationship between the average number of tumours per tumour-bearing fish and fish length, showing a gradual decrease in the number of tumours per fish as total length increases. Data based on the analysis of 510 tumour-bearing lemon sole.



decreases, with increasing fish lengths, from an average of 11.5 in the 55-59 mm group to 2.0 in the 150-154 mm group.

The relative incidences of angioepithelial nodules and epidermal papillomas with relation to fish size are illustrated in Figure 9. The data show a decrease in the relative number of AENs with increasing fish length, with a reciprocal rise in the frequency of EPs. No AENs were found on fish larger than 120 mm total length.

## Epidermal Lesions in Other Species

Scattered instances of epidermal lesions in other species of flatfish were also observed in the Strait of Georgia sampling areas. Histological examination of the tumourous tissues showed them to be identical with the lemon sole tumours. These lesions include one AEN on one starry flounder; EPs on one speckled sanddab (<u>Citharichthys</u> <u>stigmaeus</u>) and one flathead sole; and AENs, AEN/EP transitional forms, and EPs on many sand sole.

### Normal Epidermis; Light Microscopy

The morphology of the normal skin of the lemon sole is similar to previous descriptions of teleost epidermis (van Oosten, 1957; Andrew, 1959; Lagler <u>et al</u>., 1962; Romer, 1962; Hyman, 1962; Patt and Patt, 1969). The skin

### FIGURE 9

The relationship of tumour types and fish size.

Histogram illustrating the relative prevalences of AEN and EP tumour types as related to fish length. A steady drop in AEN frequency and a reciprocal rise in EP frequency are shown as total length increases. Data based on the analysis of 2965 tumours from 510 lemon sole.


consists of a simple squamous layer of epidermal cells overlying a connective tissue dermis (Figure 10). The epidermis consists of 5-7 layers of ovoid, basophilic cells; the exact number of layers varies with location on the body. Generally, the back and head regions are the thickest; the fin web epidermis is thinnest. All cells, from basal to superficial layers, are nucleate; no nucleoli are resolvable. Some flattening of the epidermal cells can be noticed in the most superficial cells; these cells also tend to be more basophilic. Darkly-staining mucus-secreting cells in various stages of maturation are interposed between the epidermal cells; the smallest mucus cells are generally located near the basal layers, while more mature cells can be observed near, and often opening to, the surface. Occasionally small, darkly-staining cells can be observed scattered throughout the epidermis, usually in the intermediate cell layers. These cells are tentatively identified as lymphocytes or resident macrophages (histiocytes).

The dermis of scale-bearing skin is composed of two layers: a superficial scale-bearing region of loosely arranged collagen fibers, fibroblasts, and pigment cells (stratum spongiosum), and a second, scale-free region of dense layers of collagen fibers (stratum compactum), which forms the boundary between the dermis and the subcutaneous tissues.

## PLATE 2 Figures 10-13

# Fig. 10

Normal skin from the mid-side of a juvenile lemon sole. Epidermis is five to seven layers thick and interspersed with mucous-secreting cells. Hematoxylin/eosin. X 160.

Fig. 11

Normal lemon sole epidermis, illustrating cells of the basal and intermediate layers. X 4520.

Fig. 12

Normal lemon sole epidermis, illustrating the relationship of mucous and epidermal cells; mucous cell is apparently ready to open to the surface. Microvilli-like pleats on superficial cells are not apparent in this section. X 4520.

### Fig. 13

Scanning electron micrograph of lemon sole epidermis, showing characteristically arranged surface pleats. X 9500.



## Normal Epidermis, Electron Microscopy

The ultrastructural morphology of the lemon sole epidermis corresponds to electron microscopic descriptions of the epidermis of other teleosts (Hendrickson and Matoltsy, 1968; Wellings and Brown, 1969; Brown and Wellings, 1970; Merrilees, 1974). The epidermis is composed of two basic cell types: squamous epidermal cells and mucussecreting cells. Mucus cells, as mentioned above, can be found randomly interposed between epidermal cells in all layers. The epidermal cells can be considered to form three distinct layers: ovoid basal cells, which immediately overlie the basement membrane; intermediate cells, which overlie the basal layer and may occur as 3-5 cell layers; and flattened superficial cells, which compose the outermost layer of the epidermis (Figures 11 and 12).

Epidermal cells in all layers can be characterized by the presence of two zones: the perinuclear zone, which contains the nucleus and all cytoplasmic organelles; and the peripheral zone, which contains many cytoplasmic filaments and ribosomes (Figure 11). Nuclei of squamous cells are irregularly shaped, often with indentations. Nucleoli were rarely observed. Cytoplasmic organelles, in the perinuclear zone of all cells, include mitochondria (usually with transverse cristae), Golgi apparatus, granular and agranular endoplasmic reticulum, and ribosomes of 15.0 nm diameter.

Differences in cellular morphology can be seen in the transition from basal to superficial layers. These changes include: a general flattening of the cells, a reduction in the complexity of cell-to-cell interdigitations, an increase in the number of desmosomes and other junctional complexes, and an increase in the number of cytoplasmic filaments. The free surface of the superficial cells is characterized by short, broad, surface pleats resembling microvilli in cross-section (Figure 13). Often the surface is covered with an amorphous or filamentous extraneous coating.

Mucus cells are found scattered throughout the epidermis at varying stages of maturity. Immature cells are generally found in the deeper layers, while mature, distended cells are found in the more superficial epidermal layers, often seen opening to the surface. The mucus cell cytoplasm contains abundant granular endoplasmic reticulum. The mucus droplets compose a large area of the central cytoplasm; most often the cytoplasmic organelles are compressed into a peripheral protoplasmic rim at the cell edges. The mucus droplets tend to be finely fibrillar in appearance and are surrounded by a single agranular limiting membrane (Figure 12).

Directly underlying the basal cell layer is a basement membrane composed of a fine fibrillar material. A layer of dermal collagen lies beneath the basement membrane. Underlying, and parallel to, these layers, are the various

pigment-containing cells. Melanophores are the commonest pigment-containing cells and are characterized by numerous, dense melanin granules in the cytoplasm. Interspersed among the melanophores are iridophores (guanidophores) which are characterized by numerous, large, empty clefts in the cytoplasm; in life, these cells contain platelike guanine crystals which are leached out during specimen preparation.

# Tumor Histology: Light Microscopy

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Three morphologic variations of the flatfish epidermal lesions can be described, each with a characteristic histological appearance (Figures 1-6). These tumor types have previously been designated as angioepithelial nodules (AENs), epidermal papillomas (EPs), and AEN/EP transition forms (Wellings <u>et al.</u>, 1964). The gross morphologic appearance of each type has been given above; histological descriptions are given below.

## Angioepithelial Nodules

The stromal component of the AEN varies from angiomous to fibromous; often a single AEN shows characteristics of each (Figure 14). The fibromous stromal type is characterized by a hyperplasia of dermal fibroblasts with an accompanying increase in collagen fibers. The hyperplasia can be seen to originate, in small AENs, in the stratum compactum, in the stratum spongiosum, or in the tissue area directly below the stratum compactum. The

PLATE 3 Figures 14-17

Fig. 14

Stroma of AEN, showing fibroma-like characteristics. Hematoxylin/eosin. X 160.

Fig. 15

Stroma of AEN, showing packed sinusoids and stromal "Xcells". Toluidine blue,  $1 \mu$  section. X 640.

Fig. 16

Cross section of lemon sole bearing AEN lesion, showing invasion of muscle tissue by the AEN stroma. Epidermis is normal. Hematoxylin/eosin. X 5.

Fig. 17

Higher magnification of lesion shown in Figure 16. Hematoxylin/eosin. X 160.



angiomous stromal type consists of many capillaries and sinusoids; usually these vascular beds show a high degree of blood cell packing. This packing of red blood cells, lymphocytes, and granulocytes becomes especially apparent in toluidine blue-stained sections of Epon-embedded material (Figure 15).

A new cell type, randomly dispersed throughout the stromal component among the fibroblasts, is found in these lesions. These cells, hereafter referred to as the unidentified stromal cell type, differ in morphology and staining characteristics from normal blood, epidermal, and dermal cell types (Figure 15).

Cells of this type are generally twice the diameter of fibroblasts and appear in hematoxylin/eosin sections as round cells with a pale red cytoplasm and a slightly darker nucleus. The similar coloring and granularity of the nucleus and the cytoplasm often make the two indistinguishable. In toluidine blue, Epon-embedded sections the cells appear as lightly staining, vacuolated cells with transparent, non-staining nucleis with prominent, darkly staining nucleoli. These cells were never observed within blood vessels but were commonly seen in angiomous and fibromous areas. They were observed most often singly, but can be found in "cords" of two to four cells. These stromal "X-cells"<sup>2</sup> differ from cell types A and B of the epidermis (described below) in size

<sup>&</sup>lt;sup>2</sup> The term "X-cell" is used collectively in referring to any of the unidentified, giant cell types. This includes stromal "X-cells", type A "X-cells", and type B "X-cells".

and staining characteristics. In some cases, the stromal component was seen to invade the underlying muscle masses, often penetrating deep among individual muscle fibers (Figures 16 and 17).

The epidermal component of the AEN varies from normal to slightly hyperplastic. In initial AENs, all epidermal cells tend to retain their normal staining characteristics. Toluidine blue sections show a marked increase in intercellular spaces, evidently due to a loosening of the cell membrane interdigitations described in the normal epidermis. This loosening of cellular contacts appears to begin in the intermediate layers of the epidermis; the superficial layers tend to retain a relatively normal appearance (Figure 18). Often the epidermal hyperplasia is accompanied by a subsequent increase in mucus cell numbers and secretions (Figure 19); more often no such increase is seen. Also characteristic of the epidermal hyperplastic stage is the appearance of a large number of small, darkly-staining cells just above the basal layer (Figure 19); these cells appear identical to the basophilic cell type observed in the normal epidermis (identified as lymphocytes or macrophages). Accompanying varying degrees of hyperplasia is the appearance of a different cell type in the epidermal component; these cells first appear along the basement membrane. They are charcterized by enlargement; an increase in the nucleus:cytoplasm ratio; the appearance of a darkly-staining, central nucleolus; loss of staining

with Feulgen's reagent; and loss of basophilia. Both nucleus and cytoplasm stain a pale pink color in hematoxylin/ eosin sections. This cell type, because of its increased diameter, will hereafter be referred to as one of the tumor's "giant cell" types; it is tentatively identified, due to identical staining characteristics, as an "X-cell" of type B (as described in EP lesions, below).

## AEN/EP Transition Forms

The stromal component of the AEN/EP transition forms (Figures 20 and 21) remains identical to that of the AEN, described above.

The epidermal component of the transition forms is characterized by thickening due to hyperplasia; with increased thickening, the epidermis becomes infolded, both over the initial stromal growth and outwards from the initial focus (Figure 20). Some of the observed hyperplastic reactions of the epidermis reach considerable proportions without further cytological changes and resemble typical hyperplastic reactions (i.e., the cells show little pleomorphism and retain normal staining characteristics and polarity) (Figures 21 and 22). Most often, increasing numbers of the ovoid, pale-staining giant cells (type B; described in the AEN epidermis) occur with epidermal infoldings (Figure 23). When these cells occur in large numbers, they tend to show increasing volume changes and decreasing stainability with hematoxylin and Feulgen's reagent (Figures 24 and 25); most

# PLATE 4 Figures 18-21

# Fig. 18

Epidermal component of AEN. Note the increase in mucous cell numbers, the increase in intercellular space in basal and intermediate layers, and the apparent vacuolation of cells in the basal layer (arrow). These vacuolated cells apparently are type B "X-cells". Toluidine blue, 1 µ section. X 640.

## Fig. 19

Section of AEN, showing stromal and epidermal components of the tissue. Note the accumulation of darkly-staining cells just above the basal layer. Hematoxylin/eosin. X 160.

#### Fig. 20

Section through AEN/EP transition form lesion. Hematoxylin/ eosin. X 5.

#### Fig. 21

Initial hyperplastic stage of AEN/EP transitional form epidermis. Mucous cells are absent. Hematoxylin/eosin. X 160.



of these cells can be classified as type A cells (see descriptions below).

# Epidermal Papilloma-like Lesions

The stromal portion of the papilloma-like lesion appears identical to the stromal component of the AEN and AEN/EP transition stages. The elements of the stroma include chromatophores, fibroblasts, collagen fibers, sinusoids, and capillaries (Figure 26). The vascular elements of this branching stroma tend to be smaller in size and show no packing of blood cells. Structures resembling altered capillaries can be found in the stroma of the papillomalike lesions; these structures presumably represent collapsed or closed capillary channels (Figure 26).

Numerous unidentified cells of the same type, identical to those previously described in the AEN stroma, can be observed in the thin stalks of the stroma of the papilloma (Figure 26).

The dominant components of the papilloma epidermis are enlarged, ovoid, "giant" cell types (Figure 27). The epidermis is composed of three cell types, each with distinctive staining characteristics and morphology. These cell types have been classified accordingly as type A (large giant cells), type B (smaller giant cells), and type C (superficial and "enveloping" cells). Staining characteristics were determined by studying paraffin-embedded sections stained with hematoxylin and eosin:

PLATE 5 Figures 22-25

#### Fig. 22

Higher magnification of tissue shown in Figure 20. Note the homogeneity of the hyperplastic epidermal cells. The average diameter of these cells is around 8  $\mu$ , the size of cells in the normal epidermis. Hematoxylin/eosin. X 640.

#### Fig. 23

Section through AEN/EP transitional form, showing the initial occurrence of type B cells (lightly stained) along the basement membrane; upper epidermal cells retain their normal appearance. Feulgen's, fast green counterstain. X 160.

## Fig. 24

Section through AEN/EP transitional stage, showing increased numbers of large, lightly staining cells in the epidermis. Larger, more central "X-cells" show staining characteristics of type A cells; type B cells can also be recognized. Hematoxylin/eosin. X 160.

#### Fig. 25

Section through EP stage lesion. Note the extreme size difference between the superficial cells (above) and the type A "X-cells" (below). Type A cells do not stain with the Feulgen reaction. Feulgen's, fast green counterstain. X 640.



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Type A. The cytoplasm of type A cells stains light red to violet and is evenly grained. The nucleus seldom appears stained; the nucleolus, however, stains darkly. The cells of type A are seldom found near the basement membrane or near the tumor surface; generally they are found in the intermediate layers of the epidermal folds.

Type B. Type B cells are characterized by a pale pink to light red cytoplasm, with a dark violet nucleus. These cells are always found in small groups along the basement membrane; rarely they can be found in the intermediate layers of the epidermal infoldings.

Type C. The cytoplasm of type C cells stains violet; the nuclei stain blue. Frequently the nuclei are swollen and the chromatin appears granular. Cells of this type occur in the superficial layers of the tumor; they also appear to fill in the spaces between the much larger giant cells (cell types A and B). For this reason, both the nuclei and the cytoplasm of these cells appear heteromorphic.

The morphologic characteristics of the various cell types found in the papilloma-like lesions were determined from a study of sections of Epon-embedded material stained with toluidine blue:

Type A: Type A cells are characteristically enlarged, irregularly shaped cells which can reach a diameter of nine times that of normal epidermal cells (Figures 26, 28 and 29). The cytoplasm generally stains lightly with

# PLATE 6 Figures 26-29

# Fig. 26

Section through EP-type lesion, showing characteristics of stromal and epidermal components. Note altered capillary structure (arrow). The average diameter of the type A "Xcells" shown is 23  $\mu$ ; compare with Figure 21. Toluidine blue, 1 u section. X 640.

#### Fig. 27

Epidermal component of EP-type lesion, showing the complete dominance of the tissue by "X-cell" types. The stromal component serves only as a supporting framework for the epidermis. Note the abundance of small, darkly staining cells near the center of the infoldings; these cells have been tentatively identified as macrophages (arrows). Hematoxylin/eosin. X 160.

## Fig. 28

Section through EP-type lesion, showing structures believed to be collapsed "X-cells" (arrows). Toluidine blue,  $l \mu$ section. X 640.

### Fig. 29

Section through superficial area of EP-type lesion, showing the characteristic structure of the outermost (superficial) cells. Toluidine blue, 1 µ section. X 640.



toluidine blue and appears heavily grained. Often the cytoplasm of these giant cells appears vacuolated; dark cytoplasmic inclusions can be observed in the larger cells. The diameter of the cells tends to increase as the center of the epidermal infolding is approached. The nucleus never stains with toluidine blue and is always greatly enlarged. A central, darkly staining nucleolus is often observed.

Type B: Cell type B appears as a smaller version of type A cells: the cells are somewhat enlarged (twice the diameter of normal epidermal cells); are rounded; possess an enlarged, round, non-staining nucleus with a darkly-staining, central nucleolus; and appear excessively vacuolated (Figure 26). These cells, as mentioned above, are most often found in groups along the basement membrane (i.e., at the periphery of the epidermal infoldings).

Type C: This cell type, as previously mentioned, can be found in the most superficial layers of the tumorous tissue as well as interspersed between the giant cells of types A and B (Figure 26). In the most superficial layers the cells appear flattened, possess finger-like connections with neighboring cells (with much intercellular space), and stain darkly with toluidine blue. No nuclei can be discerned, probably due to the intense staining characteristics of this cell type. The cells found interspersed among the giant cells of the epidermis are irregularly shaped and appear in toluidine blue sections as a darkly stained "filler".

between the lightly stained giant cells; hence, they will be referred to hereafter as "enveloping cells". Again, no nuclei can be resolved in these cells because of the intensity of the staining.

Often, in EP-type lesions, evidences of necrosis can be observed.

In toluidine blue sections, structures tentatively identified as collapsed giant cells canbe found in the central areas of the epidermal infoldings (Figure 28). These structures consist of a dense mass of heavily granular material surrounded by a clear peripheral zone. This seems to represent individual cellular necrosis. General tissue necrosis can be observed in some tumors (Figure 31). Most often (i.e., most conspicuously), necrotic tissue can be found in the lumens formed by the infolding epidermis (Figure 32). This necrotic material can be seen to consist of remnants of giant cells, identified by the presence of darkly staining nucleoli inside enlarged, transparent nuclei (Figure 33). Lymphocytes with small, darkly-staining nuclei and scanty cytoplasm can be found, often in large numbers, interspersed between the giant cells at the center of the epidermal infoldings (Figure 30).

PLATE 7 Figure 30-33

Fig. 30

Higher magnification of section through EP-type lesion shown in Figure 26, illustrating abundant, darkly staining cells, presumed to be macrophages, found in the central areas of epidermal infoldings. Hematoxylin/eosin. X 640.

Fig. 31

Section through EP-type lesion which shows evidence of general tissue necrosis. Hematoxylin/eosin. X 160.

Fig. 32

Section through EP-type lesion, showing large amounts of evidently necrotic material in a lumen formed by the infolding epidermis. Hematoxylin/eosin. X 160.

Fig. 33

Illustration of necrotic material found in an epidermal lumen, showing the presence of structures similar to remnants of "X-cells". Toluidine blue,  $1 \mu$  section. X 640.



# Tumour Histology, Electron Microscopy

#### Stroma

The stromal component of the AEN, the AEN/EP transition form, and the EP were seen to be identical in structure. For this reason the ultrastructure of the stromal tissues will be described only once. Electron microscopically, the stroma is composed of the following elements: chromatophores, capillaries, white blood cells, macrophages, fibroblasts, collagen fibers, and an unidentified cell type evidently identical to that described in the stroma of light microscopic sections.

Numerous fibroblasts can be found scattered throughout the stromal component. When located next to the epidermis, they can be noted lying parallel with the basement membrane, oriented in much the same manner as chromatophores (Figure 34). Deeper in the stroma they are heteromorphic; often narrow arm-like fibroblast processes can be observed extending into the interstitial spaces, often surrounding neighboring cells.

Collagen fibers, measuring approximately 100 nm in diameter, can be found oriented at various angles and interspersed between the cellular elements of the stroma (Figures 35, 36 and 37).

Capillaries can be observed regularly in the stroma. They generally are small (one red blood cell diameter) and exhibit a large number of pinocytotic vesicles at the cell periphery, especially toward the lumen. The cytoplasm of

## PLATE 8 Figures 34-35

Fig. 34

Electron micrograph of basement membrane area of mature epidermal lesion, showing a longitudinally-oriented fibroblast, the basement membrane, and the cells of the lesion's epidermal component (Enveloping cells and "Xcells"). X 10500.

Fig. 35

Portion of the stromal component of an angioepithelial nodule, showing some typical elements of the AEN stroma: fibroblasts, large deposits of collagenous tissue, granulocytes, stromal "X-cells", and abnormal capillary structures. X 10500.



PLATE 9 Figures 36-37

Fig. 36

Portion of stromal component of an angioepithelial nodule, illustrating some features typical of the AEN stroma: lymphocytes, fibroblasts, capillaries, macrophages, collagen, and "X-cells". X 10500.

Fig. 37

Illustration of some features of the stromal "X-cells". Note the excessive vacuolation and lack of recognizable cytoplasmic organelles. X 21000.



the endothelial cells tends to be quite electron dense; generally, mitochondria and granular endoplasmic reticulum can be observed. Often smooth muscle cells can be noted in close conjunction with the capillary (Figure 35). Altered capillaries, morphologically similar to those described in toluidine blue stained, Epon-embedded sections, can be observed (Figure 35). These appear as infolded endothelial cells.

Cells interpreted as macrophages are common in the stroma. These cells are characterized by a large, horseshoe-shaped nucleus with a periphery of condensed chromatin. The nucleoplasm is relatively electron-lucent. The cytoplasm contains many large mitochondria and membranebound, electron-dense structures of various sizes and shapes; these were interpeted as digestive vacuoles and residual bodies. The cell membrane tends to be irregular in outline and forms many finger-like processes (Figure 36).

Many small lymphocytes (Figure 36) can be observed randomly dispersed throughout the stroma. These cells are characterized by a scanty cytoplasm and an ovoid nucleus with a large amount of condensed chromatin, especially at the nuclear periphery. The cytoplasm commonly contains a few mitochondria and a few grandular endoplasmic reticulum lamellae. Another white blood cell type, interpreted as a member of the granulocyte series, was also observed. (Figure 35). These granulocytes contain a number of dense cytoplasmic granules.

The nucleus is commonly displaced to the edge of the cell; most mitochondria tend to be located in the perinuclear region.

An unidentified stromal cell type, similar in most aspects to the unidentified cell types (to be described) in the epidermal component, can be found interspersed at random between fibroblasts. These cells can be considered to be the same unidentified stromal cells described by light microscopy (Figures 15 and 26); they appear slightly enlarged or may retain the size of normal fibroblasts. They are most often observed singly, but may occur in pairs or larger The cells tend to be round or ovoid and possess groups. no intercellular connections or cell-to-cell contact of any sort. Generally the cells are enclosed by the reaches of fibroblast "arms" or partially surrounded by cells interpreted as macrophages (Figures 36 and 37). The nucleus is usually round with discrete clumps of chromatin scattered randomly throughout the nucleus; most often a central, enlarged nucleolus is present. The nucleus is surrounded by a typical double-membraned structure. Occasionally, normal-appearing granular endoplasmic reticulum can be observed in the cells' cytoplasm. Otherwise, no typical cellular organelles can be found. The cytoplasm of these cells appears heavily granulated and is relatively electron-dense. Large, double membraned vacuoles are common in the cytoplasm; these vacuoles either appear empty, appear to contain an amorphous substance, or appear to contain membrane remnants which

sometimes resemble mitochondrial cristae (Figures 35, 36 and 37). Some of these cells contain large numbers of extremely electron-dense, large inclusions similar to melanin granules (Figures 36 and 37). It is difficult to determine whether or not these particles are bounded by membrane structures. Some unidentified stromal cells contain what appear to be crystalline inclusions: spindle-shaped, electron-lucent elements generally surrounded by an area of electron density (Figure 37).

Occasionally, non-ovoid cells (Figure 38) (i.e., elongated cells oriented parallel to the basement membrane, similar to chromatophores or fibroblasts) appear excessively vacuolated in much the same manner as the rounded unidentified cell types. The irregularly shaped vacuoles are common throughout the cytoplasm; they usually appear empty or partially filled with an amorphous substance or membrane fragments.

#### AEN Epidermis

The epidermal component of the angioepithelial nodule tumor stage consists of normal epithelial cells, cells of an unknown type ("X-cells"; giant cells), and many intermediates and variations. The unidentified giant cell type can be observed interspersed among normal epidermal cells (Figure 39). The epidermal cells appear as in normal epidermis; interdigitations between neighboring cells remain complex; mitochondria, free ribosomes, and nuclei appear as normal. Interposed between (and seemingly not disrupting)

## PLATE 10 Figures 38-39

# Fig. 38

Basement membrane area of an EP-type lesion. A non-ovoid stromal "X-cell" is shown lying parallel to the basement membrane (right). Note the excessive vacuolation and resemblance to stromal fibroblasts (Figure 34), indicating a probable morphogenetic relationship between fibroblasts and stromal "X-cells". Two small epidermal "X-cells", encompassed by an enveloping cell, lie above the basement membrane (left). X 16200.

## Fig. 39

Epidermis of AEN tumour stage, showing the relationship between the initial "X-cell" types to normal epidermal cells. Note the normal-appearing plasma membrane interdigitations between neighboring epidermal cells. X 16200.



the epidermal cells are small, rounded, vacuolated cells with enlarged nuclei and central nucleoli. The cell membranes of this new cell type are closely apposed to the outer membranes of the neighboring epidermal cells. No desmosomes, finger-like processes, or other forms of cellular contacts are visible. Nuclei of cells of this type are round and enlarged; surrounded by an even, double membrane; contain discrete clumps of chromatin scattered at random throughout the nucleus; and contain large, central, dense nucleoli which appear to be composed of a heavily granular substance. Nuclear pores are visible in some cells; they appear at regular intervals, measure 100 nm in diameter, and may be associated with microtubules (Figure 40) The cytoplasm can appear extremely lucent and often seems to contain little ground substance. Large, ovoid vacuoles are common in the cytoplasm; often they are founded by a double membrane, while in other cases the membranes seem incomplete. Again, as in the analogous unidentified stromal cell type, dark electron-dense granules are present, usually without evidence of a surrounding membrane structure. These unidentified cells of the AEN epidermis appear in various stages of enlargement, cytoplasmic densities, and vacuolation (Figures 40 and 41).

This unidentified giant cell type can be found, as described above, with extremely lucent cytoplasmic substance. Ultrastructurally similar cells can also be found which tend to be extremely dense and heavily stained (Figure 41).

PLATE 11 Figures 40-41

Fig. 40

Epidermis of large AEN-type lesion. X 21000.

Fig. 41

Epidermis of large AEN-type lesion. Unidentified cell types (type B "X-cells") are found in varying stages of "balling up"; the densities of these cells varies from extremely light to dark. Several epidermal cells have retained their normal appearance. X 21000.


Normal-appearing epidermal cells can also appear densely stained; these "dark" cells possess abnormal nuclei and large cytoplasmic vacuoles (Figure 40). Those epidermal cells with normal organelle ultrastructure generally have lost the perinuclear/peripheral cytoplasmic division which normally characterize them and appear as elongated, rather than ovoid, cells. These "normal" epidermal cells tend to retain desmosomes and other junctional complexes but lose all finger-like interdigitations. Certain of these normalappearing appear in the process of losing their normal cytoplasmic density and organelles (Figure 42).

Other epidermal cells increase their cytoplasmic denisty and appear as precursors of the "enveloping cells" of the mature lesion. These cells contain many mitochondria and free ribosomes, many cytoplasmic filaments which extend across the cell along the periphery, and many desmosomes (some of which appear greatly lengthened) at the tips of the cellular "arms" (see later descriptions of enveloping cells of the EP). Occasionally these cells contain dilated endoplasmic reticulum (Figure 43).

# EP - epidermis

The epidermal component of the mature papillomalike lesion, as described by light microscopy (Figures 26-29), is composed of two general tissue areas and three basic cell types. The superficial layers of the tumor are composed of relatively normal epithelial cells (type C) with large intercellular spaces. These same normal-appearing epidermal

PLATE 12 Figures 42-43

Fig. 42

Portion of cytoplasm of a normal epidermal cell occurring in an AEN-stage lesion. This part of the cytoplasm is "sandwiched" between two "X-cell" types (top and bottom). Note the general disorganization, lack of cytoplasmic ground substance, and loss of recognizable organelles in the cytoplasm. X 19000.

Fig. 43

Epidermis of large AEN-type lesion, illustrating an enveloping cell developing among several unidentified "X-cell" types. X 16200.



cells are also present in the lower portion of the epidermal infoldings, where they compose a "matrix" in which the characteristic giant cells (types A and B) are embedded.

The superficial cells comprise the top three-tofour layers of the tumor. Characteristically, they appear as very dense, flattened cells with numerous fingerlike cellular extensions which connect them, often with desmosomes, to neighboring cells (Figures 44 and 45). The extracellular spaces are most often empty, but occasionally cellular debris, resembling the remnants of a necrotic cell, can be found (Figure 45).

The most superficial cells possess microvilli-like pleats, often coated with a fibrillar substance, similar to those found in normal epidermis (Figure 44). These cells contain numerous mitochondria and retain close cell-to-cell attachments in the horizontal plane (Figure 45). Quite often the endoplasmic reticulum of these cells is dilated and filled with an unknown, electron-dense, often heavily granular, substance (Figures 44 and 45). Nuclei of these superficial cells stain evenly dark, with little condensed chromatin. Overall, the nuclei, cytoplasm, and organelles of these cells stain with such even intensity that discrimination between cellular elements is often impossible.

Interspersed among the superficial epidermal cells small lymphocytes and histiocytes can be found. The lymphocytes are characterized by a large, chromatin-rich nucleus and a scanty cytoplasm, which commonly contains a few lamellae

PLATE 13 Figures 44-45

### Fig. 44

Superficial cell layers of a mature EP-type lesion. In this case, the section illustrates a portion of one "in-fold" of the epidermis. X 13300.

Fig. 45

Superficial cell layers of a mature EP-type lesion, showing the characteristic stellate epidermal cells, macrophages, and intercellular spaces occasionally filled with what appears to be cellular debris. The enveloping and "X-cell" types lie in the deeper portion of the lesion (to the right). X 13300.



of granular endoplasmic reticulum (Figure 45). Histiocytes (Figure 45) are characterized by a large, horseshoe-shaped nucleus with condensed peripheral chromatin. Mitochondria, endoplasmic reticulum, Golgi apparatus, residual bodies, and digestive vacuoles constitute the majority of cytoplasmic elements.

The deeper portions of the epidermal infoldings are composed primarily of giant cells enveloped by the arms of altered, heteromorphic epidermal cells of normal cytoplasmic ultrastructure. These enveloping cells are characterized by a homogenous, electron dense nucleoplasm, which sometimes shows a small degree of condensed chromatin. Grandular endoplasmic reticulum, mitochondria, and many free ribosomes often occur in the perinuclear area. The "arms" of these cells contain many free ribosomes and cytoplasmic filaments, which usually run parallel with the axis of the arm. (Figures 46 and 47). Numerous desmosomal connections occur between adjoining enveloping cells, especially at the tips of the cytoplasmic arms. No desmosomal or other junctional complexes were ever observed between the enveloping cells and the giant cells. Small lymphocytes can occasionally be found interspersed among the giant and enveloping cell types (Figure 47). The giant cells of the tumor (cell types A and B as described light microscopically) are morphologically identical in most aspects. Ultrastructural distinction between the two cell types is based on size differences and on the presence of dense cytoplasmic inclusions.

PLATE 14 Figures 46-47

Fig. 46

Deeper portion of mature EP-type lesion, showing the characteristic histologic arrangement of giant cell types ("X-cells") and enveloping cells. X 14500.

Fig. 47

Deeper portion of mature EP-type lesion (near the basement membrane), illustrating the occurrence of small lymphocytes interspersed occasionally among the "X-cell" and enveloping cell types. X 10500.



Apart from these two differences (to be described below), giant cells in the epidermal tumor component show the same pathologic characteristics. The nucleus is enlarged and ovoid, with randomly dispersed, discrete clumps of condensed chromatin; a central dense nucleolus of evenly, thickly granular material can often reach a size of one-half the diameter of the nucleus. The nucleus is surrounded by a double membrane. Nuclear pores of 100 nm diameter are commonly present at regular intervals (Figure 46). The cytoplasmic ground substance varies from dense to thinly granular. Normal endoplasmic reticulum lamellae and mitochondria are present in few cases (Figure 48). Generally the only distinguishable cytoplasmic elements present are membrane- or non-membrane-bound dense inclusions of irregular size and shape; and large, ovoid-to-round vacuoles identical to those described in the stromal and AEN unidentified cell types. Often these vacuoles can appear elongated or horseshoe shaped and may appear similar to extremely dilated endoplasmic reticulum lamellae (Figure 48). In other cases the presence of cristae-like membrane fragments located at the periphery of the vacuoles is suggestive of degenerative or dilated mitochondria (Figure 49). Occasionally bundles of microfilaments, arranged in a parallel array and bound by a single membrane, can be observed in the cytoplasm (Figure 49). The plasma membrane is free from intercellular connections and, in general, is smooth or slightly irregular. Occasionally giant cells can be found with plasma membrane infoldings

PLATE 15 Figures 48-49

Fig. 48

Epidermal component of mature EP-type lesion, showing enveloping and "X-cell" types. Note the normal endoplasmic reticulum and horseshoe- or elongate-shaped vacuoles. X 10500.

Fig. 49

Portion of cytoplasm of a type A giant cell. The cytoplasm of two enveloping cells lies on either side of the type A cell. Note the extensive plasma membrane invaginations. X 22000.



(Figures 46 and 49). Several instances of nuclear membrane "proliferation" (i.e., several layers of agranular endoplasmic reticulum closely apposed to the nuclear envelope) were noted in these cells (Figure 50). Many "X-cell" types also contained cytoplasmic "membrane whorls" (Figures 38, 49, and 51).

Light microscopy showed the presence of two types of epidermal giant cells; type B cells usually were located in groups along the basement membrane, while type A cells were generally found in the more central areas of the epidermal infoldings. Ultrastructurally, differences can be noted between those giant cells located along the basement membrane and those giant cells located in the inner tumor portions.

Ultrastructurally, those giant cells nearest the basement membrane are ovoid and range up to twice the diameter of normal epidermal cells. They tend to contain extremely large cytoplasmic vacuoles arranged in a circular fashion about the centrally located nucleus (Figures 38 and 51). These cells are similar in size and morphology to the unidentified giant cell types found in the stroma and in the AEN epidermis.

Deeper into the epidermal infoldings, the giant cells tend to be much larger (up to nine times the diameter of normal epidermal cells); they tend to be heteromorphic rather than ovoid in shape; and they tend to possess larger numbers of dense cytoplasmic inclusions (Figures 46 and 51). The electron-dense inclusions usually do not appear to be

PLATE 16 Figures 50-51

# Fig. 50

Nucleus and portion of cytoplasm of an epidermal "X-cell", illustrating a proliferation of the nuclear envelope which occasionally was observed. Note the indiscriminatively clumped chromatin. X 22000.

# Fig. 51

Low-power micrograph of epidermal portion of a mature EPtype lesion, illustrating the characteristics of type A and type B giant cells "X-cells". X 9500.



membrane-enclosed. With increasing cell size, the number of vacuoles in these larger cells seems to increase, while the average diameter of the vacuoles seems to decrease.

## Virus-like Particles

Quite frequently small virus-like particles of unknown composition or origin are present in the cytoplasm of the enveloping cells and superficial cells of the papillomalike lesions (Figures 52 and 53). In one instance they were observed in the intercellular space of the epidermis of an angioepithelial nodule (Figure 54). These particles appear round or hexagonal and measure 30 nm in diameter. The particles were never observed in typical virus crystalline arrays; most often they were found in small groups or randomly scattered throughout the cytoplasm of the enveloping or superficial cells.

PLATE 17 Figures 52-54

## Figure 52

Portion of cytoplasm of an enveloping cell found in a mature EP-type lesion, showing the presence of darklystaining virus-like particles. The particles consistently measure 30 nm in diameter. X 30000.

Fig. 53

Virus-like particles in the cytoplasm of an enveloping cell lying next to the basement membrane. X 56000.

Fig. 54

Virus-like particles found in the intercellular space of an AEN-type lesion. X 14400.



#### DISCUSSION

### Normal Epidermis

Normal epidermal structure in teleosts has been well described both light microscopically (van Oosten, 1957; Andrew, 1959; Lagler <u>et al.</u>, 1962; Romer, 1962; Hyman, 1962; Patt and Patt, 1969) and ultrastructurally (Hendrickson and Matoltsy, 1968; Merrilees, 1974). Recently the biology of the skin of the flathead sole, a flatfish often afflicted with epidermal papillomas, has been studied in some detail. Electron microscopic studies of the scales (Brown and Wellings, 1969), of the larval skin (Wellings and Brown, 1969), and of the skin of two-to-three year old fish (Brown and Wellings, 1970) have been presented.

Light and electron microscopic studies of the skin of the lemon sole showed little variation from previous descriptions of teleost epidermis. Structures interpreted in previous reports as broad, short microvilli on the surface of superficial cells were shown by scanning electron microscopy to be cross-sections of numerous surface pleats, often arranged in concentric patterns (Figure 13). This fingerprintlike cellular topography is similar to that reported in other teleost species (Jones et al., 1966; Luse and Krejsa, 1969; Merrilees, 1974). Ultrastructural observations of lemon sole guanophores correspond to previous descriptions (Setoguit, 1967); junctional complexes in the epidermis were typical

of those found in amphibian skin (Farquhar and Palade, 1965).

In an autoradiographic study of the growth of fish epidermis, Hendrickson (1967) found incorporation of thymidine in all epidermal cell layers. This indicates that mitoses are not restricted to the basal layer (as they are in other vertebrates), and that all epidermal cells participate in the maintenance of the tissue. This fundamental difference must be considered when comparing tissue reactions of fish epidermis with those of other vertebrates. No evidence of kerantinization or other differentiative processes is observed in the epidermis of most fish; instead, the epidermis is protected by a variable thickness of mucus secreted by unicellular secretory glands. Reduction or alteration of the mucus layer can result in increased susceptibility of the epidermal cells to outside agents (van Oosten, 1957).

# Biology of Epidermal Lesions

Evidence for the progression of epidermal lesions from an initial AEN stage to a mature EP lesion consists of natural history data, laboratory observations, the presence of morphologic intermediates, and histological studies.

The frequencies of occurrences of AEN and EP stages of lemon sole lesions were analyzed in terms of 5 mm-size groups. The results (Figure 9) suggest a progression from initial AENs to mature EPs: as fish size increases, the relative percentage of AENs (as compared to EPs) decreases

78.

until no AENs are found (above 135 mm). The AEN to EP transition of the flatfish lesions was postulated earlier on the basis of similar evidence (McArn, 1968; McArn <u>et al.</u>, 1968; McArn and Wellings, 1971). There appears to be a tendency, in any diseased population, for AENs to occur predominately early in the year and on younger (smaller) flatfish; EPs tend to occur almost exclusively on older (larger) flatfish, a phenomenon best explained by progressive growths of the lesions.

AENS on flatfish kept under laboratory conditions have been observed, in significant numbers, to progress to typical EP lesions (McArn, 1968; McArn <u>et al.</u>, 1968); progressive growths of the tumours were also reported by Wellings and Chuinard (1964).

The occurrence of morphologic transitional stages between AENs and EPs has been described and also seems to indicate a progressive process (Wellings <u>et al.</u>, 1964; McArn, 1968; McArn and Wellings, 1971).

The histology (light and electron microscopic) of the tumour types found in the lemon sole, and other species reported here, corresponds to previous descriptions in other species (see introduction). Most reports gave descriptions of the AEN and EP stages of tumour growth. Usually the presence of morphologic intermediates (externally) was noted, but no attempt was made to correlate histological structure with external morphological structure. Histological examination of the various tumour types (AENs, transitional forms,

and EPs) shows: 1) a distinctive histological structure for each type (i.e., a definite correlation between external morphology and internal structure, as described above), and 2) a definite pattern, involving a continuum of intermediate forms, of histomorphological changes from the AEN to EP stages. It is this pattern that will be described below, with a discussion of each stage, as a likely histologic explanation of the changes involved in the progressive growth from an initial lesion to a mature, papilloma-like lesion.

Based on correlations between histological and external morphology (presented above), the following, in simplified form, represents what I believe to be the pattern of histologic and cytologic changes occurring in the progression from initial to mature flatfish lesions (a more detailed description follows; the stages are illustrated diagramatically in Figure 55):

 hyperplasia of dermal fibroblasts, capillary growth, and an inflammatory-type response (Figures 14 and 15);

appearance of unidentified stromal cell types
("X-cells"), probably from transformation of a dermal cell
type (Figure 15; see discussion of unidentified cell types);

 hyperplasia of epidermal cells and folding of the epidermis (Figures 18, 20 and 21);

4) appearance of unidentified giant cell type(type B) along the basement membrane in the epidermal component(Figure 23);

5) appearance of type A giant cells in the interior

# FIGURE 55

Summary of the growth and structure of flatfish epidermal lesions.

The postulated growth, and structure of epidermal lesions found on juvenile flatfish. The numbered figures are explained on page 80 of the text.



of the epidermal infoldings, probably from type B precursors (Figures 24 and 25; see discussion of unidentified cell types);

6) continued increase in the numbers of cell types A and B in the epidermis until they dominate the tissue; transformation of other epidermal cells into enveloping and superficial cells (Figures 26-29);

7) necrosis of giant cell types (Figure 28);

8) consequent tissue necrosis (?) (see discussion of necrosis of tissues and death of the host fish) (Figures 31-33).

The first obvious reaction in tumorigenesis in the lemon sole skin appears to be a proliferation of dermal fibroblasts; this type of histological structure was found exclusively in the smallest AENs studied. This initial hyperplasia evidently gives rise to the stromal component of the lesion (Figure 4). This fibroblast hyperplasia resembles a fibroma, being composed mainly of fibroblasts and collagen fibers. In some tumours many blood vessels can be observed; usually these show packing of red blood cells, indicating stasis (Figure 14 and 15). Fibrosis, often accompanied by an increase in capillaries, is a common response of fish epidermis to various agent (Nigrelli and Smith, 1938, 1940; Nigrelli, 1954). Electron microscopically granulocytes (see Weinreb, 1963; Andrews, 1959), macrophages, and lymphocytes can be observed in the stroma interspersed among the fibroblasts, collagen, and capillaries (Figures 35 and 36). This would indicate some degree of

inflammatory response, as suggested by Brooks and co-workers (1969). They suggested the AEN stroma to be inflammatory in nature on the basis of red blood cells, macrophages, lymphocytes, plasma cells, eosinophil granulocytes, and vascular dilatation (with stasis) found electron microscopically. In this study, no extravascular red blood cells or plasma cells were observed in the stroma of the AENs studied. The stromal component resembles a subacute inflammatory response predominated by a fibroblastic and angioblastic proliferation (see LaVia and Hill, 1971). Nigrelli (1954) has noted the occurrence of macrophages, granulocytes, and plasma cells in response to parasitic invasions of fish. Presumably the inflammatory elements of the stromal response regress with time, leaving the fibromous framework, with vascularization, as the supporting stroma for the EP.

The unidentified stromal cell type (stromal "Xcells") can be found in most, but seemingly not all, AENs. (Figure 15). They often occur in extremely large numbers. It is not known exactly when the unidentified cell type initially appears in the lesions; examination of the earliest AEN stages revealed no cells of this type.

It appears that in some AENs the initial fibromatous reaction may be invasive in nature (Figures 16 and 17). Invasive growth of the stroma were never observed in transitional or EP stages; either the invasive growth is damaging enough to be fatal to the host at an early stage of tumour growth, or the growths are self-limiting and/or regressive.

Angiomous growths, as observed in another study (Wellings, <u>et al.</u>, 1964), often show invasive qualities without being invasive in nature.

In studies of true epidermal papillomas in other families of fish, other authors have reported initial growths similar to the angioepithelial nodule types described in flatfish. Lucke and Schlumberger (1941) found pre-papilloma lesions in other species to consist of an initial hyperemia and proliferation of dermal blood vessels. Scarpelli (1969) observed similar AEN-type to EP-type growth transitions for papillomas of the slipperydick. It has been suggested that this could be a common pattern in the growth of papillomalike growths in fish (Wellings, 1969B).

Some hyperplasia of the epidermal cells, similar to those hyperplasias noted by Nigrelli (1954) as typical responses to various parasitic stimuli in other fishes, evidently follows a varying degree of initial AEN stromal growth (Figure 21). As the epidermis thickens and begins infolding, the stromal component of the lesions becomes gradually reduced; eventually, in the mature EP, it consists of a branching series of narrow supporting "arms". Accompanying the epidermal thickening, and presumably following the hyperplasia, is the appearance of an unidentified cell type ("X-cells; giant cells), described earlier as cell type B. Usually these cells begin to accumulate along the basement membrane (Figure 23). Gradually, as the epidermis thickens and beings to infold, these cells appear larger and more

numerous (Figure 24). Staining becomes less and less intense (Figure 25). These larger cells, usually found near the center of the epidermal infoldings, exhibit different staining characteristics and were called cell type A. Electron microscopically, cell types A and B were found to be morphologically identical except for size, degree of vacuolation, and amount of cytoplasmic inclusions (see discussion below). It seems very likely, on the basis of ultrastructural morphology and time and location of the appearance of the two cell types (type B cells generally occur in late AEN or transitional forms along the basement membrane; type A cells occur in transitional and EP stages in the center of the infoldings and only, it seems, after the appearance of a large number of type B cells), that type B cells are precursors of type A cells. Eventually, in the mature lesion, these giant cell types dominate the epidermal component; they are found "embedded" in a matrix of enveloping cells (Figure 26), which also are evidently morphologically changed epidermal cells (Figure 43 and 46).

In every mature lesion studied histologically, structures interpreted as collapsed giant cells are found, usually near the very center of the epidermal infoldings (Figure 28). Generally, large numbers of cells tentatively identified as histiocytes are found in the same central regions, indicative of much phagocytic activity (Figure 30). Several mature tumors showed general necrotic tendencies throughout the tissue (Figure 31). These observations, when

considered with the general necrotic condition (to be discussed below) of the giant cell types composing the tissue, seem to indicate that necrosis is the ultimate fate of the tumors. Nigrelli and his colleagues (1965) also reported evidence of necrosis within mature and sole epidermal lesions.

The ultimate fate of the epidermal lesions or of the flatfish bearing these lesions remains uncertain. Previous studies on the natural history of this disease, as well as the present study, indicate that usually no epidermal lesions are found on flatfish over 200 mm total length. Evidently certain factors are operating in the removal of tumourous tissues from fish, or in the selected removal of tumourbearing fish from the population.

When the frequencies of tumour-bearing lemon sole are expressed as percentages per 5 mm size group, a definite rise and fall of tumour prevalence is noted as fish length increases. This phenomenon was noted in previous studies of the natural history of this disease (Good, 1940; Nigrelli <u>et al.</u>, 1965; Wellings <u>et al.</u>, 1965; McArn and Wellings, 1971; Miller and Wellings, 1971; Mearns and Sherwood, 1974). An initial increase in the relative prevalence of tumourbearing fish occurs until the 75-79 mm group, where the incidence approaches 50%. After this peak, the gradual decrease in tumour prevalence (to a zero point in the 160-164 mm group) indicates one (or both) of two things: 1) the lesions are regressing or are being sloughed-off the fish surface at a fairly constant rate, and/or 2) tumour-bearing

fish are being removed from the population at a much faster rate than are non-tumour-bearing fish.

The main evidence that the lesions are rejected or lost (due to necrotic processes) is the histological presence of cellular and tissue necrosis, as observed in this study in lemon sole EPs and in sand sole EPs by Nigrelli and his co-workers (1965). Kelly (1971), in a study of epidermal papillomas on lemon sole in Puget Sound, Washington, reported sloughing or rejection of tumors under laboratory conditions at a rate of 70% over a two month period. In other studies (Wellings et al., 1964; Miller and Wellings, 1971), tumors were reported to progress in the laboratory; no evidence of necrosis or tumor regression was found. This contradictory evidence is possibly due to size differences, and consequently tumor maturity, of the fish selected for study. Mature tumors would seem more likely to show evidence of necrosis or regression than would young, progressive tumor stages. There are at least two instances of regression or rejection of tumor-like growths in other families of fish. Fish pox, a hyperplastic epidermal disease, appears to be self-limiting and is probably controlled by temperature and/or the season. Fish pox lesions have been observed to undergo regression (Lucke and Schlumberger, 1948). N. Peters (personal communication) has observed that European eels, afflicted with cauliflower-like papillomas, often reject tumorous tissues.

Evidence that the drop in tumor frequency with increasing size is caused by the death of tumor-bearing fish

is inconclusive. Analyses of the average number of lesions per fish with relation to size (Figure 8) shows an irregular, but definite, decline as fish length increases. This phenomenon was shown in the present study of lemon sole as well as in earlier studies (Wellings <u>et al.</u>, 1965; Miller and Wellings, 1971). Presumably, if the tumors are not being sloughed-off or rejected, then the absence of lesions in fish over 180 mm in length must indicate that the disease is fatal. If this is the case, then heavily-infected fish would be expected to be removed from the population at a faster rate than others; this would manifest itself in a decrease in the average number of tumors per fish as fish size increases.

Part, if not all, of this decrease can be explained by observations indicating that very often the spreading edges of many AENs merge to form a single large EP. Cooper and Keller (1969), in their study of lesions on lemon sole in San Francisco Bay, presented evidence which indicated that the disease had no lethal effects on the population.

Miller and Wellings (1971), on the other hand, have shown a marked reduction in growth rates of tumor-bearing flathead sole in the second year of life, as compared with normal fish; this indicates that the lesions have a detrimental effect on the health of the fish. Wellings and his co-workers (1964) found that it could not be shown that the rate of disappearance of tumorous fish from the population exceeded that of normal fish. Although no definite statement can be made concerning the fate of the lesions or of fish bearing the lesions, it seems certain that some flatfish

must die from the affliction of EPs. Although no metastases or truly invasive growths were observed, fatalities could be caused by indirect effects of the disease, of which there are many possibilities. The position of lesions probably has an obvious effect on the health of the fish, especially when they occur about the gills, eyes, or mouth. Fin tumors could be expected to cause sluggishness and would decrease the fish's ability to escape predators. Any debilitating effect on the health of the flatfish is likely to increase the susceptibility of the fish to other agents (e.g. bacterial infections), predators, or harsh environmental conditions.

Possibly, both factors (fish death and tumour necrosis and loss) are at work in the removal of numbers of tumour-bearing flatfish from the population. Conceivably, survival of tumour-bearing fish long enough for the tumours to mature, and necrose, is requisite for the phenomenon of tissue necrosis and sloughing-off.

The nature of the unidentified cell types ("Xcells") found in the stroma and epidermis of flatfish skin lesions remains, to some extent, uncertain. Two hypotheses have been proposed (Brooks <u>et al.</u>, 1969): 1) the cells are unicellular parasitic organisms, and 2) the cells are transformed fish cells. The unidentified cells were postulated to be unicellular parasites because: 1) the morphology of the cells is similar, in some ways, to protozoans, 2) the unidentified cells are often found, in the stroma, in cords of two to four, 3) the cells are usually surrounded by a

50 nm wide cell coat, 4) the stroma shows evidence of an inflammatory response, 5) collagen and fibroblast processes often encircle the cells, and 6) macrophages can be seen in the process of engulfing the unidentified cells. No cell coats were observed around the cells in the present study. The cells studied in the lemon sole showed no real resemblance to any described unicellular organism (see Pitelka, 1963). although other workers reported a resemblance to a haplosporidian parasite of fish as described by Perkins in 1968 (McArn et al., 1968). The unidentified cell type of the lemon sole possessed no evidently functional cytoplasmic organelles; those cells which did, appeared to be transitions between normal epidermal cells and the new cell type. The initial appearance of this new cell type evidently occurs, in the stroma, after the angioblastic and fibroblastic proliferation and, in the epidermis, after an initial typical hyperplasia. These observations suggest that these unidentified cells occur as a result of, and not as a cause of, the tissue reactions characteristic of the disease. The inflammatory nature of the AEN stroma possibly could be elicited by these cells if they underwent antigenic changes due to transformation or infection, similar to the phenomenon of virus-induced tumor antigens.

Cellular hypertrophic reactions in fish cells of various species have been shown to be elicited by several agents (Weissenberg, 1949); these agents include viruses (Nigrelli and Ruggieri, 1965; Weissenberg, 1965), Rickettsia

(Wolke, 1970), bacteria (Davis, 1953), fungi, and parasitic protozoans (Nigrelli and Smith, 1940; Nigrelli, 1948b; Sprague, 1968; Weissenberg, 1968; Lom, 1970; Trager, 1974). Ultrastructural studies of the unidentified cell types in flatfish tumors showed no similarities in morphology to any of the above agents, or to any cytopathological changes (except hypertrophy) elicited by these agents.

Histologic evidence presented in this thesis indicates that the unidentified stromal and epidermal cells found in flatfish lesions probably are transformed fish cells, rather than protozoan parasites; the transformed cells differ from the normal in that they are extremely hypertrophied and show characteristics of extreme and irreversible cellular This conclusion is based on: 1) the absence of any dmage. evidence indicative of protozoan or other parasitic invasion, intracellular or extracellular, and 2) observations of a number of morphological intermediates between normal epidermal cells and the giant cell types found in the lesions. The following discussion illustrates what I have concluded to be a likely progression of cytopathic events involved in the transformation of normal cells to giant cell types.

Probably the first reaction of the epidermal cells is a loss of cell-to-cell contact, which in some cases appears to result in an increase in intracellular space and in the formation of stellate or "prickle" cells, a condition often noted in histological studies of hyperplastic tissues (Figure 44). Presumably the cells then lose desmosomal contact and

"ball up" (Figure 41). Vacuolation of the cell apparently follows, as does loss of cell volume regulation. The increasingly severe vacuolation is correlated with the disappearance of all cytoplasmic organelles (Figures 41 and 42). Very often the vacuoles tend to be elongated, horseshoe-shaped structures which resemble dilated endoplasmic reticulum (Figure 48); other larger, more rounded vacuoles often contain membrane remnants and resemble dilated, degenerated mitochondria. The matrix of such vacuoles is clear; membranes resembling cristae tend to be extremely small, irregular, and located at the vacuole periphery (Figure 49). These observations tend to suggest that these "mitochondria" (Wellings et al., 1967; Brown et al., 1969) are non-functional, degenerative structures. The cytoplasmic vacuoles can be found bounded by either a single or a double (the more common) membrane, presumably depending on their origin (Figures 48 and 49). On the basis of the above observations, I believe these vacuoles to be the product of damaged and dilated mitochondria and endoplasmic reticulum. Often membrane "whorls" occur in the cytoplasm, another indication of cellular damage (Figures 38 and 49). The cytoplasmic ground substance tends to become irregularly amorphic and may become extremely light (Figure 46). The nuclei of giant cell types in mature lesions tend to be enlarged; chromatin is always clumped into discrete, random clumps, nucleoli are enlarged, prominent, and consist exclusively of ribosome-like particles (Figure 46). These observations tend to indicate degenerative processes; the
cytopathic changes described are changes to non-functionality and ultimate necrosis (Trump and Ginn, 1969; Trump and Arstila, 1971). It is important to note here that the subcellular pattern of necrosis of flatfish "X-cells" differs from the pattern of necrosis found in other cells in reaction to lethal injuries to the cell. In particular, necleolar disintegration and karyolysis, common changes in necrotic cells (Ginn <u>et al.</u>, 1968), do not occur in flatfish tumour cells. This would suggest that the necrotic changes described in flatfish cells are not the result of a non-specific lethal injury to the cell (e.g. anoxia), but may instead be the result of cellular transformation (i.e., changes in the genome) or of a specific subcellular injury caused by a specific aetiological agent.

These changes are characteristic of both the hypertrophic epidermal cell types as well as the analogous stromal cell type. Cells classified as type A giant cells show these characteristics; however, they have undergone further hypertrophy and vacuolation than have the smaller, type B, giant cells. They are characterized by the appearance of large numbers of extremely electron-dense cytoplasmic inclusions of unknown origin and composition (Figure 46 and 51). Brooks and his co-workers (1969) indicated that if these unidentified cell types were transformed fish cells, then motility of such cells must be required, since the cells are found in both stromal and epidermal components of the lesion. This hypothesis need not be the case. Staining properties and ultrastructural studies of the two cell types indicate certain

differences between the epidermal and stromal unknown cells. These differences might arise if the origin of the cell types were different: a degenerative, necrotic disease process presumably would be capable of causing similar cytopathic effects in stromal fibroblasts, for instance, and in epidermal cells. The stromal unidentified cell type was never observed to undergo extreme cellular hypertrophy to the extent that its analogous epidermal cell type can. The origin of the stromal cell type is unknown; an obvious hypothesis might suggest an origin from fibroblasts, but no concrete evidence whatsoever has been observed.

There is doubt as to whether the flatfish epidermal lesions should be considered as hyperplastic or neoplastic processes (Wellings et al., 1968; Mawdesley-Thomas, 1972). There might also be doubt as to whether the lesions represent a hypertrophic process with compensatory hyperplasia of superficial and enveloping cells, or whether the lesions are true hyperplastic or neoplastic reactions preceding a secondary, and incidental, cellular hypertrophy. Discussion or speculation without further studies would be inconclusive at best. Some workers (Wellings et al., 1965; Miller and Wellings, 1971) believed the lesions to be true neoplasms. Nigrelli and his co-workers (1965) postulated that the lesions were hyperplastic in nature. The difference between hyperplasia and neoplasia usually cannot be determined by histological studies, as noted in the case of fish pox (Schlumberger and Lucke, 1948). According to the definition

of neoplastic hyperplasia (Prehn, 1972) as: "that form of hyperplasia which is caused, at least in part, by an intrinsic inheritable abnormality in the involved cells", animal neoplasms must be transferrable by the inoculation of living neoplastic cells. Transplantation studies done on flatfish tumors have all proved negative (Good, 1940; Chuinard and Wellings, 1964; McArn, 1968; Wellings, 1969).

The actiology of epidermal papilloma-like lesions afflicting Pacific coast flatfish remains an enigma. Many factors may be involved in the genesis of any disease process; probably this multiple factor approach applies to flatfish lesions. Evidence of virus-like particles present in the cells of mature epidermal lesions of lemon sole was given above; the relationship of these particles to the disease process, if any, has not been determined.

Viruses have been proven to be the cause of one cellular hypertrophic disease of fish epidermis, lymphocystis disease (Weissenberg, 1965; Wolf <u>et al.</u>, 1966), and have been implicated as a probable cause of several other epidermal hyperplastic diseases in fish. These latter include fish pox (Nigrelli, 1952; Mawdesley-Thomas and Bucke, 1967), the cauliflower disease of eels (Koops <u>et al.</u>, 1970), and other hyperplastic reactions of fish epidermis (Walker, 1966 and 1968). Electron microscopic evidence of virus-like particles has been given in this report (Figures 52-54) as well as in other reports of studies of flatfish epidermal lesions (Wellings <u>et al.</u>, 1964, 1965 and 1967;

McArn, 1968; Kelly, 1971). The use of viral techniques in isolation or transmission of the virus has proved negative (Wellings <u>et al.</u>, 1965; McArn, 1968; Wellings, 1969).

From the evidence presented in this thesis, it seems reasonable to conclude that: 1) epithelial tumours in Pacific coast flatfish progress from initial angioepithelial nodules to mature epidermal papilloma-like growths, and 2) this progression is manifest in histological and cytological changes, as documented above. Other evidence presented in this thesis suggests that the unidentified "X-cells" which compose the mature tissue are transformed, necrotic fish epidermal cells, rather than intra- or extracellular parasitic protozoans (as has been hypothesized). Evidence for the progression of normal epidermal cells into "X-cells" is primarily the presence of morphologic intermediates, the timing of appearance of the new cell type in the tissue, and the lack of resemblance of the "X-cells" to any known protozoan. Further work must be done to definitely establish this fact. The full biological nature of the lesions (i.e., whether the growths are the result of hyperplastic, neoplastic, or hypertrophic processes) is unknown, and further work is certainly needed and desirable. Autoradiographic studies of the various tumour stages would show where (by which cells) hyperplastic or neoplastic growth was occurring. Further, and more extensive, observations of tumour-bearing fish under laboratory conditions may be useful in determining the hyperplastic or neoplastic nature of the lesions. The

nature of flatfish tumours is presently unclear and the similitude of these growths and true epidermal papillomas seems questionable. Certainly the histomorphology of the flatfish lesions differs sufficiently from other known papillomas, carcinomas, or other diseases of fish epidermis to warrant further study.

## LITERATURE CITED

- Angell, C.L. and Miller, B.S. (1974), Ecology of Tumor-bearing English Sole (Parophrys vetulus) in Puget Sound, Washington. J. Fish Res. Bd. Can. (submitted). (Cited by Mearns and Sherwood, 1974).
- Andrew, W. (1959), A covering called skin. In: <u>A textbook</u> of Comparative Histology. New York: Oxford University Press.
- Arnowitz, O., Nigrelli, R.F. and Gordon, M. (1951), A spontaneous epithelioma in the platyfish, <u>Xiphophorus (Platypoecilus)</u> <u>variatus</u>. Zoologica, N.Y. 36:239.
- Brooks, R. E., McArn, G.E. and Wellings, S.R. (1969), Ultrastructural observations on an unidentified cell type found in epidermal tumours of flounders, J. Natn. Cancer. Inst. 43:97.
- Brown, G.A. and Wellings, S. R. (1969), Collagen formation and calcification in teleost scales. Z. Zellforsch, 93:571.
- Brown, G. A. and Wellings, S. R. (1970), Electron microscopy of the skin of the teleost, <u>Hippoglossoides elassodon</u>. Z. Zellforsch 103:149.
- Chuinard, R.G., Wellings, S.R., Bern, H.A. and Nishioka, R.S. (1964), Epidermal papillomas in pleuronectid fishes from the San Juan Islands, Washington, Fedn. Proc. Fedn. Am. Socs. Exp. Biol. 23:337.
- Coates, C.W., Cox, R.T., and Smith, G.M. (1938), Papilloma of the skin occurring in an electric eel, <u>Electrophorus</u> <u>electricus</u> L. Zoologica, N.Y. 23:247.

Cooper, R.C. and Keller, C.A. (1969), Epizootiology of papillomas in English sole, <u>Parophyrs vetulus</u>. Natn. Cancer Inst. Monogr. 31:173.

- Davis, H.S. (1953), Culture and diseases of fishes, Berkeley, University of California Press.
- Deys, B.V. (1969), Papillomas in the Atlantic eel, <u>Anguilla</u> <u>vulgaris</u>, Natn. Cancer Inst. Monogr. 31:187.
- Farquhar, M.G. and Palade, G.E. (1965), Cell junctions in amphibian skin. J. Cell Biol. 26:263.
- Forrester, C.R. (1969a), Life history information on some groundfish species. Fish Res. Bd. Can. Tech. Rep. 105:17pp.
- Forrester, C.R. (1969b), Results of English sole tagging in British Columbia waters. Pac. Mar. Fish. Comm. Bull. 7:10pp.
- Ginn, F.L., Shelburne, J.D. and Trump., B.F. (1968), Disorders of cell volume regulations. I. Effects of inhibition of plasma membrane adenosine triphosphatase with ouabain. Am. J. Pathol. 53:1041.
- Good, H.V. (1940), A study of an epithelial tumour of <u>Parophyrs</u> <u>vetulus</u>. Master's dissertation, University of Washington. Harold, E.S. and Innes, K.F. (1922), The shrimp and associated organisms of San Francisco Bay. Steinhart Aquarium, Calif. Acad. Sci., Golden Gate Park, San Francisco, (cited in Mawdesley-Thomas, 1972).
- Hart, J.L. (1973), <u>Pacific fishes of Canada</u>. Fish Res. Bd. Can. Bull. 180:740pp.

Hendrickson, R.C. (1967), Incorporation of tridiated thymi-

dine by teleost epidermal cells. Experientia 23:357.

- Hendrickson, R.C. and Matoltsy, A.G. (1968), The fine structure of teleost epidermis. I. Introduction and filamentcontaining cells. J. Ultrastr. Res. 21:194. II. Mucous cells. J. Ultrastr. Res. 23:213.
- Hoffman, G.L., Dunbar, C.E., Wolf, K. and Zwillenberg, L.O. (1969), Epitheliocystis, a new infectious disease of the bluegill, <u>Lepomis machrochirus</u>. Antonie van Leeuwenhoek, J. Micro. Serol. 35:146.
- Honma, Y. and Kon, T. (1968), A case of the epidermal papilloma in the witch flounder from the Sea of Japan. Bull. Jap. Soc. Scient. Fish. 34:1.
- Hyman, L.H. (1962), The comparative anatomy of the skin and the exo-skeleton. In: <u>Comparative Vertebrate Anatomy</u>, Chapter VI, p. 79. Chicago, Chicago University Press.
- Imai, T. and Fujiwara, N. (1959), An electron microscopic study of a papilloma-like hyperplastic growth on a goby, <u>Acanthogobius flavimanus</u>. Kyushu J. Med. Sci. 10:135.
- Jones, M.R., Holliday, F.G.T., and Dunn, A.E.G. (1966), The ultrastructure of the larvae of the herring (<u>Clupea</u> <u>harengus</u>) in relation to the rearing salinities. J. Mar. Biol. Ass. U.K. 46:235.
- Karlsson, U. and Schultz, R.L. (1965), Fixation of the central nervous system for electron microscopy by aldehyde perfusion. I. Preservation with aldehyde perfusates <u>vs</u> direct perfusion with 0s04, with special reference to

membranes and the extracellular space. J. Ultrastr. Res. 12:160.

- Kelly, D.L. (1971), Epidermal papilloma in the English sole, <u>Parophrys vetulus</u>. PhD dissertation. University of California at Berkeley (cited by Mearns and Sherwood, 1974).
- Ketchen, K.S. (1947), The age, growth, and mortality of the lemon sole (<u>Parophrys vetulus</u> Girard) on the British Columbia fishing grounds. M.A. thesis. University of British Columbia.
- Ketchen, K.S. (1953), Tumourous infection in sand soles of Northern Hecate Strait (British Columbia). Report on the survey of Hecate Strait in July, 1953. M.S., 3pp. (cited by Mawdesley-Thomas, 1972).
- Ketchen, K.S. and Forrester, C.R. (1955), Migrations of lemon sole (<u>Parophrys vetulus</u>) in the Strait of Georgia. Fish. Res. Bd. Can. Pac. Prog. Rep. 104:11.
- Kimura, I., Sugiyama, T. and Ito, Y. (1967), Papillomatous growth in sole from Wakasa Bay area. Proc. Soc. exp. Biol. Med. 125:175.
- Koops, H., Mann, H., Pfitzner, I., Schmidt, O.J. and Schubert, G. (1970), The cauliflower disease of eels. Spec. Pubs. Am. Fish. Soc. 5:291.
- Lagler, K.F., Bardach, J.E., and Miller, R.R. (1962), <u>Ichthyology</u>. New York, John Wiley and Sons.
- Levings, C.D. (1967), A comparison of the growth rates of the rock sole, Lepidopsetta bilineata Ayres, in northeast

Pacific waters. Fish. Res. Bd. Can. Tech. Rep. Ser. No. 36:43pp.

- Lom, J. (1970), Protozoa causing diseases in marine fishes. Am. Fish. Soc. Sp. Publ. No. 5:101.
- Lucke, B. (1938), Studies on tumours in cold-blooded vertebrates. A Rep. Tortugas Lab. 1937-1938:92.
- Lucke, B. and Schlumberger, H.G. (1941), Transplantable epithelioma of the lip and mouth of catfish. I. Pathology. Transplantation to anterior chamber of eye and into cornea. J. Exp. Med. 74:397.
- Lucke B. and Schlumberger, H.G. (1949), Neoplasia in coldblooded vertebrates. Physiol. Rev. 29:91.
- Luft, J.H. (1961), Improvements in Epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9:409.
- Lumann, M. and Mann, H. (1956), Beobachtungen uber die Blumenkohl Krankheit der Aale. Arch. FischWiss. 7:229.
- Luse, S.A. and Kresja. R.J. (1964), A correlative ultrastructural and scanning electron microscopic study of the golden guppy (Poecilis reticulata). Anat. Rec. 163:220.
- Manzer, J.I. (1951), Growth in lemon sole in Northern Hecate Strait. Fish. Res. Bd. Can. Pac. Prog. Rep. 86:13. Mawdesley-Thomas, L.E. (1972), Some tumours of fish.

Diseases of Fish. Symp. Zool. Soc. London No. 30:191. Mawdesley-Thomas, L. E. and Bucke, D. (1967), Fish pox in the roach (Rutilus rutilus L.), Vet. Rec. 81:56.

In:

- McArn, G. (1968), The natural history and pathology of skin tumors found on pleuronectids in Bellingham Bay, Washington, PhD. thesis. University of Oregon Medical School.
- McArn, G., Chuinard, R.G., Miller, B.S., Brooks, R.E. and Wellings, S.R. (1968), Pathology of skin tumors found on English sole and starry flounder from Puget Sound, Washington. J. Natn. Cancer Inst. 41:229.
- McArn, G. and Wellings, S.R. (1971), A comparison of skin tumors in three species of flounders. J. Fish. Res. Bd. Can. 28:1241.
- Mearns, A.J. and Sherwood, M. (1974), Environmental aspects of fin erosion and tumors in southern California dover sole. Tran. Am. Fish. Soc. 103:799.
- Merilees, M.J. (1974), Epidermal fine structure of the teleost <u>Esox americanus</u> (Esocidae, Salmoniformes). J. Ultrastr. Res. 47:272.
- Miller, B.S. (1969), Life history observations on normal and tumor-bearing flathead sole in East Sound, Orcas Island, Washington. PhD. thesis. University of Washington.
- Miller, B.S. and Wellings, S.R. (1971), Epizootiology of tumors in flathead sole (<u>Hippoglossoides elassodon</u>) in East Sound, Orcas Island (Washington). Trans. Am. Fish. Soc. 100:247.
- Nigrelli, R.F. (1948a), Hyperplastic epidermal disease in the bluegill sunfish, <u>Lepomis macrochirus</u> Rafinesque. Zoologica, N.Y. 33:133.

- Nigrelli, R.F. (1948b), Prickle cell hyperplasia in the snout of the redhorse sucker (<u>Moxostoma aureolum</u>) associated with an infection by the myxosporidian <u>Myxobolus moxostomi</u> sp. nov. Zoologica, N.Y. 33:43.
- Nigrelli, R.F. (1952), Virus and tumors in fishes. Ann. N.Y. Acad. Sci. 54:1076.
- Nigrelli, R.F. (1954), Tumors and other atypical cell growths in temperate freshwater fishes of North America. Trans. Am. Fish. Soc. 83:262.
- Nigrelli, R.F., Ketchen, K.S. and Ruggieri, G.D. (1965), Studies on vrisu diseases of fishes. Epizootiology of epithelail tumors in the skin of flatfishes of the Pacific coast, with special reference to the sand sole (<u>Psettichthys melanosticus</u>) from northern Hecate Strait, British Columbia, Canada. Zoologica, N.Y. 50:115.
- Nigrelli, R.F. and Smith G.M. (1938), Tissue responses of <u>Cyprinodon variegatus</u> to the myxosporidian parasite, <u>Myxobolus lintoni Gurley.</u> Zoologica, N.Y. 23:195.
- Nigrelli, R.F. and Smith, G.M. (1940), A papillary cystic disease affecting the barbels of <u>Ameiurus</u> sp. Zoologica, N.Y. 25:89.
- Nigrelli, R.F. and Ruggieri, G.D. (1965), Studies on virus diseases of fishes. Spontaneous and experimentaly induced cellular hypertrophy (Lymphocystis disease) in fishes of the New York Aquarium, with a report of new cases and an annotated bibliography (1874-1965). Zoologica, N.Y. 50:83.

Oota, K. (1952), An epidermic occurrence of tumor-like hyperplasia of epidermis in a species of fish, <u>Acanthogobius</u> <u>flavimanus</u>. Gann. 43:264.

- Pacis, M.R. (1932), An epithelial tumor of <u>Parophrys</u> <u>vetulus</u>. Master's dissertation. University of Washington.
- Patt, D.I. and Patt, G.R. (1969), Comparative Vertebrate Histology. New York: Harper and Row.
- Perkins, F.O. (1968), Fine structure of the oyster pathogen <u>Minchinia nelsoni</u> (Haplosporidia, Haplosporiidae). J. Invert. Path. 10:287.
- Pitelka, D. (1963), <u>Electron Microscopic Structure of Protozoa</u>. New York: Pergamon Press.
- Prehn, R.T. (1972), Neoplasia. In: <u>Principles of Pathobiology</u> (M. LaVia and R. Hill, eds.), p. 191. New York: Oxford University Press.

Rae, B.B. (1965), The Lemon Sole. London: The Whitefriars Press.

- Reynolds, E.S. (1963), The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17:208.
- Romer, A.S. (1962), <u>The Vertebrate Body</u>. Philadelphia: W.B. Saunders.
- Russell, F.E. and Kotin, P. (1957), Squamous papilloma in the white croaker. J. Natn. Cancer Inst. 18:857.
- Sabatini, D.D., Bensch, K. and Barnett, R.J. (1963), Cytochemistry and electron microscopy--the presence of cellular ultrastructure and enzymatic activity by aldehyde fixation. J. Cell Biol. 17:19.

Scarpelli, D.G. (1969), (Quoted in Wellings, 1969).

Schlumberger, H.G. and Lucke, B. (1948), Tumors of fishes,

amphibians, and reptiles. Cancer Res. 8:657.

- Setoguti, T. (1967), Ultrastructure of guanophores. J. Ultrastruct. Res. 18:324.
- Sjostrand, F.S. (1967), <u>Electron Microscopy of Cells and</u> Tissues. Vol. 1. New York: Academic Press.
- Smith, G.M. (1935), A hyperplastic epidermal disease in the winter flounder infected with <u>Cryptocotyle lingua</u> (Creplin). Am. J. Cancer 25:108.

Smith, J.G. and Nitsos, R.J. (1969), Age and growth studies of English sole, <u>Parophrys vetulus</u>, in Monterey Bay, California. Bull. Pac. Mar. Fish Comm. 7:73.

- Sprague, V. (1969), Microsporidia and tumors, with particular reference to lesions associated with <u>Ichthyosporidium</u> sp. Schwartz 1963. Natn. Cancer Inst. Monogr. No. 31:237.
- Steeves, H.R. III (1969), An epithelial papilloma of the brown bullhead, <u>Ictalurus nebulosus</u>. Natn. Cancer. Inst. Monogr. No. 31:215.
- Stolk, A. (1953), Tumors of fishes. III. Carcinoma of the epidermis in the black variety of the viviparous cyprinodont <u>Xiphophorus hellerii</u> Heckel. Proc. K. Ned. Akad. Wet. (C) 56:143.
- Stolk, A. (1956), Tumors of fishes. XI. Carcinoma of the skin in the anabantid <u>Colis labiosa</u> (Day). Proc. K. Ned. Akad. Wet. (C) 59:624.
- Stolk, A. (1958), Tumors of fishes. XXII. Epidermoid carcinoma of the upper lip in the characid Ephippicharax

orbicularis (Valenciennes). Proc. K. Ned. Akad. Wet. (C) 61:201.

- Stolk, A. (1960), Tumors of fishes. XXXI. Epidermoid carcinoma
  in a strain of the cichlid <u>Etroplus maculatus</u> (Bloch).
  Proc. K. Ned. Akad. Wet. (C) 63:200.
- Taylor, F.H.C. (1946), Lemon sole spawning grounds in Baynes Sound. Fis. Res. Bd. Can. Pac. Prog. Rep. 68:48.

Tavolga, W.N. (1951), Epidermal fin tumors of the gobiid fish, <u>Bathygobius soporator</u>. Zoologica, N.Y. 36:273.

- Trager, W. (1974), Some aspects of intracellular parasitism. Science 183:269.
- Trump, B.F., and Arstila, A.U. (1972), Cellular and subcellular reactions of cells to injury. In: <u>Principles of Patho-</u> <u>biology</u> (M. Lavia and R. Hill, eds.), p. 9. New York: Oxford University Press.
- Tr mp, B.F. and Ginn, F.L. (1969), The pathogenesis of subcellular reaction to lethal injury. Methods Achiev. Exp. Pathol. 4:1.
- van Cleve, R. and el-Sayed, S.Z. (1969), Age, growth and productivity of an English sole (<u>Parophrys vetulus</u>) population in Puget Sound, Washington, Bull. Pac. Mar. Fish. Comm. 7:52.
- van Oosten, J. (1957), The skin and scales. In: <u>The Physiology</u> of Fishes (Brown, M.E., ed.), Vol. 1:27. New York: Academic Press.

Walker, R. (1966), Pers. comm., cited in Wolf, 1966.
Walker, R. (1969a), Epidermal hyperplasia in fish: two types
without visible virus. Natn. Cancer Inst. Monogr. No.
31:209.

Walker, R. (1969b), Virus associated with epidermal hyper-

plasia in fish. Natn. Cancer Inst. Monogr. No. 31:195. Weinreb, E.L. (1963), Studies on the fine structure of teleost

blood cells. I. Peripheral blood. Anat. Rec. 147:219. Weissenberg, R. (1949), Cell growth and cell transformation

induced by intracellular parasites. Anat. Rec. 103:517. Weissenberg, R. (1965), Fifty years of research on lympho-

cystis virus disease of fishes (1914-1964). Ann. N.Y. Acad. Sci. 126:362.

- Weissenberg, R. (1968), Intracellular development of the Microsporidian <u>Glugea anomala</u> Moniez in hypertrophying migratory cells of the fish <u>Gasterosteus aculeatus</u> L. An example of the formation of Xenoma tumors. J. Protozool. 15:44.
- Wellings, S.R. (1969a), Neoplasia and primitive vertebrate phylogeny: Echinoderms, prevertebrates and fishes--A review. Natn. Cancer. Inst. Monogr. No. 31:59.

Wellings, S.R. (1969b), Environmental aspects of neoplasia in fishes. In: <u>Fish in Research</u> (Neuhaus, O.W. and Halver, J.E., eds.), p. 3. New York: Academic Press.

- Wellings, S.R. and Brown, G.A. (1969), Larval skin of the flathead sole, <u>Hippoglossoides</u> <u>elassodon</u>. Z. Zellforsch 100:167.
- Wellings, S.R. and Chuinard, R.G. (1964), **Ep**idermal papillomas with virus-like particles in the flathead sole, <u>Hippo-</u> <u>glossoides elassodon</u>. Science 146:923.

- Wellings, S.R., Chinard, R.G. and Bens, (1965), A comparative study of skin neoplasms in four species of pleuronectid fishes. Ann. N.Y. Acad. Sci. 126:479.
- Wellings, S.R., Chinard, R.G. and Cooper, R.A. (1967), Ultrastructural studies of normal skin and epidermal papillomas of the flathead sole, <u>Hippoglossoides elassodon</u>. Z. Zellfrosch Mikrosk. Anat. 78:370.
- Wellings, S.R., Chinard, R.G., Gourley, T. and Cooper, R.A. (1964), Epidermal papillomas in the flathead sole, <u>Hippoglossoides elassodon</u>, with notes on the occurrence of similar neoplasms in other pleuronectids. J. Natn. Cancer Inst. 33:991.
- Willis, R.A. (1967), <u>Pathology of Tumours</u>. 4th Ed., London: Butterworth.
- Wolf, K. (1966), The fish viruses. Adv. Virus. Res. 12:35.
- Wolf, K., Gravell, M., Malsberger, R.G. (1966), Lymphocystis virus: isolation and propagation in Centrarchid fish cell lines. Science 151:1004.
- Wolke, R.E., Wyand, D.S. and Khairallah, L.H. (1970), A light and electron microscopic study of epitheliocystis disease in the gills of Connecticut striped bass (<u>Morone saxatilis</u>) and white perch (<u>Morone americanus</u>). J. Comp. Path. 80:559.
- Young, P.H. (1964), Some effects of sewer effluents on marine life. Calif. Fish Game 50:33.