NATURAL HISTORY AND PATHOLOGY OF SKIN TUMORS FOUND ON PLEURONECTIDS IN BELLINGHAM BAY, WASHINGTON

by

Glynn E. McArn, M.S., M.P.H.

A THESIS
Presented to the Department of Microbiology
and the Graduate Division of the University of Oregon Medical School
in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

June 1968

APPROVED:

[redacted]

(Professor in Charge of Thesis)

[redacted]

(Chairman, Graduate Council)

TO

DR. S. R. WELLINGS and DR. CHARLES E. GARDNER

This thesis would not have been possible

without their continued support and encouragement.

THE SOLES*

by

David Wagoner

The soles are lying in shallows off Dungeness Spit.
They rest on vacant sides and stare at the sun.
Their skin like sand is glowing against the sand.

The tide has come and gone. It comes again.
The soles are lying still as their own breath.
The ocean passes through the straits of their gills.

One eye has moved an inch in a million years
To join the other on the burning side,
Drawn up like a moon from underlying night.

They dart and bury themselves as we drift over.
They cloud the sand across their speckled halves.
Their fixed, their wandering eyes stare up again.

*From *The Saturday Review*, May 7, 1966. By permission of the author and publishers.

TABLE OF CONTENTS

INTRODUCTION

STATEMENT OF THE PROBLEM

Epidermal papillomas have been observed in epizootic proportions in a number of species of pleuronectid fishes (flounders) in the waters of Puget Sound and the San Juan Islands (1, 2, 3, 4, 5, 6, 7). Pacis (1), in 1932, and Good (2), in 1940, collected tumored English sole, *Parophrys vetulus*, from several sites in the lower Sound. In 1964, Wellings *et al.* (3, 4) described the frequent occurrence of epidermal papillomas on flathead sole, *Hippoglossoides elassodon*, from East and West Sounds of Orcas Island. In 1965, Nigrelli, Ketchen, and Ruggieri (5) made similar observations relative to the sand sole, *Psettichthys melanostictus*, and the rock sole, *Lepidopsetta bilineata*, collected from northern Hecate Strait, British Columbia, Canada. More recent reports by Chuinard, Berkson, and Wellings (6), in 1966, and McArn *et al.* (7) indicate that English sole and starry flounders, *Platichthys stellatus*, collected from Port Gardner, Port Susan, and Bellingham Bay are affected in large numbers with a similar disease.

Epizootics in fishes consumed by man and animals are of considerable concern from the standpoint of public health, as well as being of general interest from a basic biological aspect. The natural history of epizootic skin tumors in flounders has been partly described in only one species, the flathead sole (8). It would be of considerable importance to determine whether or not the disease is similar in other flounder species.

The purpose of this thesis, therefore, is to describe the biology, natural history, and pathology of epidermal papillomas in the English sole and starry flounder, two species in which the disease has not been studied in detail. Also included will be a description of attempts to isolate viral or other biological agents from the tumors. It is believed that the descriptive and zoological approach to the disease is a necessary prelude to additional laboratory studies planned to establish the etiological agent involved.

REVIEW OF THE LITERATURE

The phylum Chordata is customarily separated into four subphyla: the Hemichordata (acorn or tongue worms), the Urochordata (ascidians, tunicates, or sea squirts), the Cephalochordata (amphioxus), and the Vertebrata (mammals, birds, reptiles, amphibians, fishes, elasmobranchs, and cyclostomes) (9).

There are no published reports of neoplasms in the Hemichordata and the Urochordata. There is only one report of a neoplasm in a cephalochordate: a chromaffinoma of the intestine in *Branchiostoma lanceolatum* (amphioxus) (10). The relative absence of tumors in these subphyla may be more apparent than real, because little effort has been made to find tumors, and because few specimens are collected and examined, due to commercial unimportance. There is therefore insufficient data upon which to base a judgement as to the prevalence of neoplasia among the prevertebrate subphyla.

In the subphylum Vertebrata, representatives of all classes are affected with tumors. The number of reports for some classes is

considerably greater than for others. Among the fishes, tumors are most frequently observed in economically valuable members of the Families Salmonidae, Cyprinidae, Gadidae, Bothidae, and Pleuronectidae. It is unlikely that members of these families are more susceptible, but rather that the high incidence reflects the fact that fishes of these families are caught in large numbers for food, and, therefore, tumors are more likely to be noticed.

Skin Tumors in Fishes:

There are many reports of spontaneous tumors among the teleosts (bony fishes). Teleosts are more numerous and inhabit more of the earth than any other class of vertebrate, and are economically important to man. Therefore, neoplasia is more likely to be observed than in some other groups of animals. Recorded tumors among the elasmobranchs (sharks, dogfish, rays) are accordingly much rarer, presumably because of their smaller numbers and infrequent use as human food items.

Neoplasms of both epithelial and mesenchymal origin are common in fish (11). Representatives of most of the epithelial tumors--papillomas, adenomas, odontomas, adenocarcinomas, and the thyroid tumors--have been reported. Papillomas are the most common benign tumors, and the epitheliomas (epidermoid carcinomas) are the most common malignant tumors of epithelium, occurring most frequently on the lips, oral mucosa, and skin. Tumors of melanocytic origin, arising in the skin, subcutaneous tissue, and eyes, are found more frequently in fish than in birds or mammals, perhaps because fish possess greater numbers of these cells per unit area of skin. Pigment cells other than

melanocytes which may give rise to tumors are erythrophores, xanthophores, and guanophores.

The earliest report of a skin tumor in a fish was that of McFarland (12) in 1901. The tumor was found on the lower lip of a catfish, *Ictalurus catus*, and was a lobulated papillomatous growth measuring 4 cm in diameter. Multiple small nodules were also present on the mucosa of both lips and the surrounding skin. Microscopic examination of the lesions revealed epithelial cells supported by an avascular connective tissue stroma. The large lesion was invasive and was classified as an epithelioma (squamous cell carcinoma).

Dauwe and Pennemann (13), in 1904, reported two cases of epitheliomas in aquarium carp, *Cyprinus carpio*. Tumors developed on the dorsum of the head of both fish. The lesions were identical microscopically and consisted of squamous epithelial cells that formed pegs, the centers of which were necrotic.

In 1905, Bashford, Murray, and Cramer (14) described an epithelioma in another carp, *Cyprinus carpio*. The location of the tumor was not given, but the authors commented on the striking similarity of this lesion to squamous cell carcinomas of mammals. Attempts to transplant neoplastic tissue by multiple inoculations into six carp of the same species gave negative results.

Papillary tumors of the lip on barbels, *Barbus fluviatialis*, caught in the Mosel River, Germany, were described by Keysselitz (15) in 1908. The solitary lesions were pea-sized, round, and rarely extended to the adjacent epidermis. Microscopic examination revealed closely packed epithelial cells supported by a coarse connective tissue stroma which

extended into the tumor from the corium and which was frequently infiltrated by leukocytes. The nuclei of many of the epithelial cells contained one or more inclusion bodies. The author suggested a developmental sequence in the appearance of the inclusions, and compared the inclusions to those of vaccinia.

Murray (16), in 1908, reported an instance of a cone-shaped mass on the tail of a male stickleback, *Spinachia spinachia*. Histologically, the tumor was classified as a squamous cell carcinoma, arising from the skin. The neoplastic cells were invasive, involving both the myotomes and the vertebral column. Ulceration and central necrosis were present.

In several species of gudgeon, *Gobio*, Schroeders (17) described "fibro-epitheliomata." The lesions were single or multiple, yellow in color, sharply circumscribed, and papillomatous.

Small, multiple, wart-like papillomas were described by Fiebiger (18) on a group of climbing perch, *Anabas scandens*, kept in the same aquarium for two years. Various parts of the body were involved, and the lesions varied in size from a poppy seed to a pea. Histologic examination revealed marked hyperplasia of the epidermis with papillary proliferation of the connective tissue of the corium; leukocytes infiltrated the corium. The author noted the similarity of these lesions to the infectious warts of mammals.

Another epithelioma was reported by Clunet (19), in 1910. The tumor was located on the lower lip of a barbel, *Barbus vulgaris*, and was the size of a hazelnut. Invasion of the floor of the mouth had occurred. Mitoses were numerous and there was a suggestion of epithelial pearl formation.

Johnstone (20), in 1912, described a cauliflower-shaped growth on the snout of a 120-lb halibut, *Hippoglossus hippoglossus*. The lesion was on the pigmented side of the fish and portions of it appeared darker than the normal epithelium. Grossly, the tumor was irregular and lobulated, and varied from closely packed folds in some areas to a minutely papillated surface in others. Histologically, there were coarse, branching and anastomosing fibers which extended into the interior of the papillae. Epidermal and epithelial components were absent, and the limiting membrane was indistinct. The papillae were very vascular and consisted of fine areolar networks with nuclei and bundles of coarse fibers. A tendency toward possible local invasion was suggested.

Two croakers, *Pogonias chromis*, with lip tumors were examined by Beatti (21) in 1916. A cauliflower-shaped lesion was found on the upper lip of one specimen. This animal also had a papillomatous lesion on its forehead. Hyperplasia of the epidermis and the connective tissue of the cutis was noted, with only one area where epithelial pegs had penetrated an otherwise continuous basement membrane. In the second fish the lower lip was involved, and there was frank invasion of the subcutaneous tissue by epithelial pegs. Both of these lesions were interpreted as papillary epitheliomas with early invasion.

Breslauer (22), in 1916, described a papillary tumor which principally occurred on the lips and occasionally on the oral mucosa or fins of stint, *Osmerus eperlanus*. Thirty-seven affected fish, taken from the brackish waters of an inlet of the Baltic Sea, were examined. The tumors were frequently located in opposition to each other along the occlusive surfaces of the lips, and varied in size from a few

millimeters to large cauliflower-like masses which were the size of the fish's head. Histological sections revealed typical epidermal papillomas. In the majority of cases no evidence of invasion was seen, but occasionally the arrangement of cells suggested early local malignancy. Cytoplasmic inclusion bodies were periodically observed.

A 1 x 1-cm epithelioma on the right side of the mandible of a whiting, *Merlangus merlangus*, was described by Johnstone (23). Microscopically, it was a typical papilloma with extensive proliferation of the epithelial pegs; the basement membranes were intact.

Three types of tumors on two species of *Gobio*, a small marine fish, were reported by Anitschkov and Pavlovsky (24) in 1923. The first type was a small nodule or flattened lesion; the larger of these were distinctly papillomatous. The bases of these tumors and the neighboring corium were sites of inflammation. The authors pointed out the similarity of this lesion to chronic inflammatory papillary hyperplasia of the skin and mucosa of man. The second type of tumor, found on *Gobio nigronatatus*, was an isolated flat growth. On section, the typical benign papillomatous structure was seen without evidence of inflammation. The third type was a 2 x 1 x 1-cm, cauliflower-shaped tumor on the dorsum of a *Gobio blennoides*. Histologically there was marked irregular hyperplasia of epidermal cells which were supported on a fine connective tissue stroma. There was no invasion; the authors classified the tumor as a papillary carcinoma.

Johnstone (25), in 1925, described flat "cutaneous warts" on the body surfaces of three plaice, *Limanda limanda*, caught in the North Sea. A mature female had a white, 6 x 5-cm lesion located on the pigmented

(ocular) side. The lesion was raised in the center and thinned out toward the periphery. A second mature female had white, 2 to 3-mm nodules scattered over the pigmented side. Confluent growth was observed in two locations near the center of the body. The third specimen (a mature male) presented two white tumors: one measured 10 x 5 mm and covered the right eye; the other was 2 cm in length and was located on the dorsal fin. The lesions on all three of these fish were histologically similar and were classified as papillomas. A brief mention was made of an identical lesion located on the pectoral fin of a small plaice, *Pleuronectes platessa*. Approximately twelve warts, white in color and averaging 5 mm in diameter, were distributed over the non-pigmented side of the fish. The main elements were layers of epidermal cells supported by a stroma of delicate connective tissue.

In each of two goldfish, *Carassius auratus*, Sagawa (26) described wart-like tumors the size of peas on each pectoral fin. The epithelium was hyperplastic and thrown into papillary folds. Keratinization was present in some portions of the lesions. Clavate and mucous cells, present in normal skin, were absent in the tumor.

In 1929, Takahashi (27) observed a crucian carp, *Carassius carassius*, for sixteen months. The fish had a sharply circumscribed greyish-white lesion on its operculum which measured 3 x 2 x 0.5 cm. Microscopically the tumor showed evidence of active epithelial proliferation with both pearl formation and numerous mitotic figures. The amount of stromal connective tissue was minimal. The lesions were not invasive, nor were there any metastases.

epithelioma, perhaps caused by inflammation.

Pacis (1), in 1932, observed tumors, which were apparently epidermal papillomas, in a total collection of 4,500 English sole, *Parophrys vetulus*. Tumor fish usually measured 9 to 20 cm in length, and the majority (73.6%) were in their second year of growth.

A large (32 cm) brown trout, *Salmo trutta*, observed by Thomas (32), had a tumor at the posterior margin of the operculum. The tumor measured 22 x 11 x 5 mm, and was poorly circumscribed and typically papillomatous, both grossly and microscopically.

Smith (33), in 1935, described a hyperplastic epidermal disease, morphologically similar to papilloma, in winter flounders, *Pseudopleuronectes americanus*. This disease was associated with encysted metacercariae of the trematode, *Cryptocotyl lingua*, a member of the family *Heterophyidae*. Sections of the tumor consisted of a hyperplastic epidermis supported on an edematous corium. Fibrous bands were seen to extend from the corium into the epithelium. The corium was heavily vascularized and mucous cells were located in the epidermis.

In 1938, Coates, Cox, and Smith (34) described a papilloma located on the right mid-dorsal region of a 6-ft adult electric eel, *Electrophorus electricus*. This specimen was caught in the Amazon River and maintained in the New York Aquarium for over four years. The lesion was observed as it grew from a small elevated pinkish-grey structure a few millimeters in diameter, until it was excised ten weeks later, at which time it measured 2 x 2.5 cm. Histologically, the growth was a pedunculated mass composed of squamous epithelium containing mucous

cells. The epithelium was supported by a fibrous core of connective tissue.

Thirty tumor-bearing "slippery dicks", *Iridio bivittata*, were found among 6,000 of these fish examined at the Dry Tortugas, Florida (35). The tumors on this tropical fish were grey-white, flattened, nodular, sometimes massive elevations of the skin. The lesions occurred principally on the lateral surfaces and fins, sometimes destroying the latter. Microscopically, a delicate corium was covered by many layers of epithelial cells. The epithelial cells extended into the corium, but invasion into deeper tissues was not observed. The epithelial cells of the tumor were characteristically larger than normal epidermal cells. The question of whether these lesions were true neoplasms or non-neoplastic hyperplasia was left unanswered.

Good (2), in his master's thesis on epidermal papillomas of English sole, *Parophrys vetulus*, reported 61 tumor fish out of a total collection of 1,355 (4.50%). The fish were collected over a two-year period in the Seattle, Washington area, utilizing a beach seine set at 100-200 feet offshore. The fish ranged in total length from 6.8 to 19.0 cm. Most (75%) of the 89 tumors found on 61 fish were solitary; in the remainder there were up to eleven tumors per fish. The majority of the tumors were located on the pigmented (ocular) side of the fish. Grossly, the tumors were typical papillomas. Microscopically, the epithelial cells possessed hyperchromatic nuclei, and stromal invasion was present. A number of the tumor fish were observed in the laboratory for periods as long as fifteen weeks in order to determine the fate of the lesions. Ten of twelve tumors continued to enlarge; two regressed. No

new tumors appeared during the period of observation. The author was unable to transplant the tumors to normal fish of the same species. Attempts to induce tumors with 20-methylcholanthrene failed.

Papillary cystic formations were observed on the barbels of a catfish, *Ameiurus nebulosus* (LeSueur) (36). The lesions were interpreted as a response to infection by the myxosporidian *Henneguya thelohan*, which filled the cysts. Microscopically, the cysts were encapsulated by fibrous tissue, and the overlying epithelium was thickened, giving the tumor a warty appearance. Mucous cells and giant dermal gland cells were also present.

A common transplantable epithelioma of the lips and mouth of a catfish, *Ameiurus nebulosus*, was described by Lucké and Schlumberger (37). The tumors occurred as large masses of tissue which originated from the lips and dental plates, occasionally involving other parts of the mouth and skin. Histologically, the tumors consisted of epithelial cells in papillary arrangement, supported by a stroma of delicate, vascularized connective tissue. Frequently the larger lesions invaded neighboring normal tissue and blood vessels. Intense hyperemia of the dermis occurred initially and preceded neoplastic proliferation; no abnormal epithelial changes were noticed at that time. Within a period of two weeks the overlying epithelium began to thicken and became elevated. By two months a noticeable nodule was seen.

Of 148 autotransplants into the anterior chamber of the eye, 71.4 per cent were successful. Heterotransplants into the anterior chamber of the eye of goldfish and frogs failed. Autotransplants to the cornea

were successful 85.7 per cent of the time, while homotransplants were only successful in 11.9 per cent of cases.

Nigrelli (38), in 1946, described a fibroepithelial growth on the head of one ocean pout, *Macrozoarces americanis*. The reddish, rugose, spongy lesion extended from the interorbital region to beyond the border of the maxilla. The lesion was grossly papillomatous and resembled the fibroepithelial growth of the marine turtle, *Chelonia mydas*, described by Smith *et al.* (39). Histologically, the epithelium varied in thickness from a few to several cell layers, and keratinization was absent. The bulk of the tumor consisted of vascularized corium. The etiology was unknown, although the author suggests that trauma may have played a role.

A tumor frequently occurring on eels (*Anguilla anguilla*) collected in the waters between Denmark and Sweden, and in the Baltic Sea, was described by Christiansen and Jensen (40) in 1947. The large cauliflower-like growths were located on the head in the majority of cases. Microscopically, the lesions were epidermal papillomas. Attempts to transplant the tumors failed.

Two large tumors affecting a common sucker, *Catostomus commersonnii*, were observed by Schlumberger and Lucké (41). The large 8-year-old fish had tumors located on its right side; one measured 6.1 x 5.5 x 4.0 cm and involved the tail fin. The smaller tumor measured 20 mm in diameter and was located on the caudal peduncle. Grossly, both were firm, raised, lobulated masses which appeared to be papillomas. Sectioned material revealed a delicate stroma supporting a massive layer of epithelial cells. The more basal cells were tall

columnar in shape, and nuclei were prominent, containing one or more nucleoli. Mucous cells and clavate cells were present in the epithelium.

Prickle cell hyperplasia of the snout of a redhorse sucker, *Moxostoma aureolum*, was reported by Nigrelli (42) in 1948. Numerous myxosporidians, *Moxostomi*, were located deep in the dermis in large cysts, and may have stimulated the development of the delicate fibrous networks which surrounded spore masses and trophozoites. Mild inflammation was present. Connective tissue and epithelium were hyperplastic and sometimes papillary. The author suggested that proteolytic enzymes and other chemical substances elaborated by the myxosporidians could be responsible for the cell degeneration, hypertrophy, and hyperplasia.

An endemic hyperplastic epidermal disease similar to carp pox was reported in the bluegill sunfish, *Lepomis macrochirns* (Rafinesque), by Nigrelli (43) in 1948, in fish captured from a lake near New Preston, Connecticut. Over a four-year period, 200 fish were collected with papillomatous skin nodules. Microscopic sections showed extensive hyperplasia of epithelial cells supported on a central core of connective tissue. Ciliates, *Trichodina* and *Ichthyophthirus*, parasitized the skin. The *Ichthyophthirus* were deeply embedded in the epithelium. Attempts to induce this disease in normal fish failed. The author suggested that the parasites were toxic to the host, or that the host was sensitive to mechanical irritants which produced the disease.

Aronowitz, Olga, and Nigrelli (44) described a single case of an epithelioma in an adult female platyfish, *Xiphophorus variatus*. The

lesion was located on the dorsolateral surface of the head above the operculum and seemed to be an epidermoid carcinoma. The tumor measured 5.25 x 5.00 x 5.25 mm, was smooth, pink, and sharply circumscribed. Histologically, the subepidermal portion showed evidence of pearl formation without cornification. The epidermis was thickened, but otherwise normal. Invasion of neighboring tissue had occurred.

Tavolga (45), in 1951, described multiple epidermal tumors of the fins of four gobiid fish, *Bathygobius soporator*, collected at Bimini Island, British West Indies. The lesions varied in size from 1 x 1.5 mm to large, lobulated growths protruding from the body and completely replacing fin structures. Microscopically, the smaller lesions revealed epidermal hyperplasia. As the growths enlarged, epithelial cords penetrated the underlying tissue. The largest lesion was typically epitheliomatous with infiltration, pearl formation, and many mitotic figures. The tumors were highly vascularized with blood vascular sinusoids and fluid-filled vesicles.

An example of a hormonally induced epithelial hyperplasia in a goldfish, *Carassius auratus*, was reported by Ghadially and Whiteley (46) in 1952. Treatment of immature and mature male and female goldfish with testosterone proprionate resulted in the appearance of papillomatous hyperplasia of the gills and pectoral fins. Fifty per cent of the treated females failed to respond, while all males responded. The lesions were similar to the papillomatous structures that normally appear on the pectoral fins of mature male, but not female, goldfish during the breeding season. The fin lesions were indistinguishable from ordinary papillomas. The lesions of the gills had a mammillary

appearance. Microscopic examination of a "bramblehead" goldfish showed a hyperplastic epidermis with myxomatous degeneration of the stroma. Attempts to produce tumors in both warm and cold water fish with 9,10-dimethyl-1,2-benzanthracene were unsuccessful.

In 1952, Nigrelli (47) reported the development of papillomas on three of several climbing perch, *Anabas scandens*, in the New York Aquarium. The author mentioned the similarity of these lesions to those described by Fiebiger (18), who compared them to infectious warts. The lesions occurred on the lips, fins, and operculum. No inclusion bodies were seen in sectioned material, and attempts to transmit the disease were unsuccessful.

This author also reported, in the same publication, papillomas of the lips in dwarf gourami, *Colisa lalia*. A high incidence (0.5-1%) of tumors were observed on fish in outdoor breeding ponds in Florida. The epidemiology of this disease seemed to indicate an infectious process. Histologically, the tumors were epidermal papillomas; cartilage and bone formed in the stroma in many instances. Some tumors were interpreted as malignant papillary epidermoid carcinomas. Inclusion bodies were evident, but attempts to transmit the disease failed.

The occurrence of five epidermal papillomas in a *Koi* fish, *Anabas testudenius* (Bloch), was reported by Sarkar and Dutta-Chaudhuri (48) in 1953. The specimen was caught in a large *bheri* (a shallow stagnant embanked water area) near Calcutta. The tumors were cauliflower-shaped, scattered over various parts of the body, and varied in size from 3 x 3.2 cm to 0.75 x 0.3 cm. Microscopically, the growths were typical epidermal papillomas composed of folds of epidermal cells supported on

branching stalks of connective tissue. The epithelial cells were well differentiated and no invasion was seen.

Stolk (49) in 1953 reported 11 specimens of the black variety of viviparous cyprinodont, *Xiphophorus hellerii*, with epidermal carcinomas originating at the base of the tail and infiltrating the corium, subcutaneous tissue, and muscle. The neoplastic cells were pleomorphic and invasive. There was an accompanying growth of melanophores.

In 1954, Ermin (50) described an ocular tumor in an interspecific hybrid resulting from a cross between an *Anatolichthys splendens* and *A. transgrediens*. The tumor was responsible for the protrusion of the eye. Histologically, there was infiltration of the muscles and skeletal elements of the orbit. The author interpreted the neoplasm as an epithelioma (epidermoid carcinoma). Internal mucus secretion of the tumor was found to contain gram positive bacteria-like organisms.

An instance of an invasive epithelioma (*i.e.* epidermoid carcinoma) in an adult female cat shark, *Scylliorhinus catulus*, was reported by Stolk (51) in 1956. The broad sessile lesion occurred on the oral mucosa of the lower jaw behind the lip. Histologically there were closely packed epithelial cells supported by a well vascularized stroma.

In the same year, Stolk (52) also reported carcinomas of the skin in the anabantid, *Colis labiosa* (Day). Six males of the same litter had broadly sessile, lobulated masses on the skin of the trunk and caudal peduncle. The neoplastic epithelial cells formed pegs and strands which invaded the underlying connective tissue stroma. The tumor cells were polyhedral in shape and possessed hyperchromatic nuclei. The stroma contained fibrocytes, melanoblasts, melanocytes, and melanophores.

Squamous cell papillomas were observed by Russell and Kotin (53) on ten of 353 white croakers, *Genyonemus lineatus*, caught in polluted waters off the coast of California. The tumors were multiple, small, firm, white, and located about the mouth. Microscopically, they consisted of papillary epithelial layers supported on a connective tissue stroma. There was no invasion. Nonkeratinized epithelial cells were occasionally seen in "pearl" formation. There were no tumors on fish of the same species collected from adjacent unpolluted waters.

Epidermoid carcinomas were described in the characid, *Ephippicharax orbicularis*, by Stolk (54) in 1958. The tumors were found on the upper lip of a number of adult fish of both sexes. Microscopically, there were strands and masses of slightly pleomorphic tumor cells, arranged in papillary pegs and separated by a vascularized stroma of connective tissue. The nuclear chromatin network was delicately structured. The author felt that the neoplasm was invasive, but no metastases were observed.

An electron microscopic study of a papilloma-like hyperplastic skin growth in gobies, *Acanthogobius flavimanus*, was discussed by Imai and Fujiwara (55). The fish were caught during 1957 and 1958 in and around the Bay of Hakata, Kyushu, Japan. The author acknowledges the similarity of the tumors to those described by Fiebiger in the climbing perch (18), in the goldfish by Sagawa (26), and in the sucker by Schlumberger *et al.* (41). The lesions were grey to grey-red, soft, and either flat or typically raised and papilloma-like. Histologically, marked proliferation of the epidermal components was accompanied by papillary growth of connective tissue. There was no invasion. By electron

microscopy, round cytoplasmic particles were found, generally in cells undergoing degenerative changes. These particles were compared to the virus of human molluscum contagiosum.

In 1960, Stolk (56) described epidermoid carcinomas occurring on five cichlids, *Etroplus maculatus* (Bloch). The large, crater-shaped tumors were variously located on the external surfaces and varied in size from 16 x 8 x 3 mm to 29 x 18 x 5 mm. Histologically, there were closely packed polyhedral epithelial cells supported by a delicate, heavily vascularized connective tissue stroma. The nuclei of the tumor cells were pleomorphic with coarse chromatin clumps. Infiltration of the corium and musculature by long pegs and strands of epithelial cells was evident, and necrosis was occasionally seen. The author considered these tumors to be "species-specific", and perhaps sex-linked.

The pike-perch, *Stizostidion vitreum*, is affected by two types of skin warts: 1) the virus-induced lymphocystis disease which causes cell hypertrophy, and 2) multiple dermal neoplasms associated with another virus. In the second type of tumor, there are compact nodules of spindle cells, associated with a variable quantity of vascular elements and fibrous support. Walker (57) described the neoplastic cells by electron microscopy. The cytoplasm was rich in ribosomes, there was margination of nuclear chromatin, and there were numerous virus-like particles. The virus-like particles were in most respects unlike the virus of lymphocystis disease. They measured approximately 1000 Å in diameter, capsules were incompletely polyhedral, and there was a dense central core. Complete virus particles were found extracellularly and

in the cytoplasm, but not in the nucleus. Incomplete particles showed continuity with the cell membrane system, such as is observed in the instance of the Bittner agent and some other tumor viruses (58).

In a series of recent papers, Wellings and co-workers (3, 4, 59, 60, 61, 62) and Nigrelli, Ketchen, and Ruggieri (5) described the frequent and widespread occurrence of epidermal papillomas on several species of pleuronectids (flounders) collected in the waters of northern Puget Sound, the San Juan Islands, and British Columbia. The species affected included the flathead sole, *Hippoglossoides elassodon*, the sand sole, *Psettichthys melanostictus*, the rex sole, *Glyptocephalus zachirus*, the rock sole, *Lepidopsetta bilineata*, the dover sole, *Microstomus pacificus*, the starry flounder, *Platichthys stellatus*, and the English sole, *Parophrys vetulus*. More recently, Kimura, Sugiyama, and Ito (63) reported a similar disease on one *Limanda herzensteini* and four *Hippoglossoides dubius*, two species of flounders occurring in Japanese waters. Still more recent data reported by McArn *et al.* (7) describes identical tumors in the starry flounder and English sole. Unpublished data* indicate that rock sole and Pacific halibut, *Hippoglossus stenolepis*, collected in the Bering Sea, also sometimes carry similar tumors. The last observation is of importance because these northern waters are relatively unpolluted by sewage, industrial wastes, *etc.*

The incidence of the disease is strikingly high, as indicated in the selected data in Table 1.

---

*Wellings, unpublished.

TABLE 1

Skin Tumors of Flatfishes*

Author	Species	Number of Tumor Fish Reported	Total Number of Fish Collected	% Tumor Incidence	Reference Number
Wellings, *et al.* 1964	*Hippoglossoides elassodon*	281	4,364	6.4%	3
	Psettichthys melanostictus	2	Unknown	--	
	Glyptocephalus zachirus	3	Unknown	--	
	Parophrys vetulus	2	Unknown	--	
Wellings, *et al.* 1965	*H. elassodon*	336	5,250	6.4%	59
	G. zachirus	3	Unknown	--	
	P. vetulus	2	Unknown	--	
	P. melanostictus	2	Unknown	--	
Chuinard, *et al.* 1966	*P. vetulus*	61	1,377	4.4%	61
	Platichthys stellatus	38	780	4.9%	
Nigrelli, *et al.* 1965	*P. melanostictus*	231	729	31.7%	5
	Lepidopsetta bilineata	5	Unknown	--	
Kimura, *et al.* 1967	*Limanda herzensteini*	1	Unknown	--	63
	Hippoglossoides dubius	4	Unknown	--	
McArn, *et al.* In press	*P. vetulus*	93	1,792	5.2%	7
	P. stellatus	57	1,063	5.4%	

* Not an inclusive table.

The tumors in most reports were typical cauliflower-like epidermal papillomas, composed of folds of thickened epidermis supported on branching fronds of stroma containing collagen, small blood vessels, and melanophores. The epidermal component contained hypertrophied, ovoid cells with prominent nucleoli, which were interpreted as transformed epidermal cells (3, 5). In the instance of the flathead sole (3) and probably also the English sole and starry flounder (62), the earliest and smallest discernible lesion occurred on fish in the first year of life. This lesion, descriptively named "angioepithelial nodule", consisted of a 1-to-2 mm, slightly raised, hemispherical proliferation of vascular connective tissue capped by a layer of mildly hyperplastic epidermis. Toward the end of the first year of life, the angioepithelial nodules were observed to transform by a series of intermediate stages into typical epidermal papillomas (3).

Electron microscopy of the tumors of flathead sole (4, 60) revealed the presence of several categories of cytoplasmic bodies of possible viral nature. Abundant virus-like particles of identical morphology to Coxsackie virus (64) were observed in both the stromal and epithelial cells of one tumor of a starry flounder (7).

Skin Tumors in Amphibia:

There are only a few reports of spontaneous or experimentally induced skin tumors occurring in amphibians. Instances of neoplasia have been reported in both orders of Amphibia: urodeles (salamanders, newts) and anurans (frogs).

The first report of a skin tumor in a urodele was that of Murray (65) in 1908. In a crested newt, *Triton cristatus*, there was a mammillated mass at the angle of the jaw and multiple nodules scattered over the tail. The surface of each lesion was pitted, the pits leading to tubules which passed downward and through the full thickness of the skin. Although the tumors were not invasive, the author interpreted them as adenocarcinomas of the skin glands.

The first suggestion of a viral-induced tumor in a urodele came from the work of Champy and Champy (66) in 1935. The authors studied a carcinoma of the skin of the newt, *Triton alpestris*. This tumor was first observed in 1931 when three out of five newts of the same species simultaneously developed cutaneous tumors. Histologic examination revealed glandular formations of polyhedral and cylindrical cells, and the tumors were invasive. The removal of tumorous animals, and the periodic addition and removal of three groups of fresh animals of the same and different species into the contaminated waters, resulted in the development of more tumors, 1.5 to 2 years later, in three of four *T. alpestris*. These four animals were members of the first groups added to the contaminated water; all other groups containing the same and different species of animals failed to develop the lesions. Furthermore, the lesions were transmissible from tumorous newt to normal newt by implantation of tumor fragments. However, the recipients, which had never been in the contaminated water, developed tumors at sites distant from the implantation sites.

In order to establish that a tumor has a genetic basis for its development, it is necessary to conduct controlled breeding experiments.

Sheremetieva-Brunst and Brunst (67) found a male and a female grey axolotl, *Siredon mexicanum*, each with small black spots on its body. These spots gradually increased in size and became melanotic tumors. Mating of the two animals resulted in the appearance of similar tumors on some of the offspring. The cell of origin was the melanophore, and the tumor was invasive in some cases. These investigators were successful in transmitting the melanoma to apparently normal axolotls.

Rickenbacher (68) observed an ulcerated carcinoma on the head of a female newt, *Triturus alpestris*. There was invasion of the skin, but no metastases. The tumor was interpreted as an adenocarcinoma originating from skin glands.

A well vascularized tumor, interpreted as an epithelioma, was observed on the upper jaw of an adult black axolotl, *Siredon mexicanum*, by Sheremetieva-Brunst (69). The axolotl became emaciated and died two months later. Fragments of the tumor were transplanted to 21 homologous animals; 18 developed tumors. Pieces of tumor tissue transplanted into albino axolotls increased in size in 85% of cases.

Stolk (70) observed multiple fibromas on six adult newts, *Triturus taeniatus*, all from the same litter. The author described four phases in the development of the lesion: a) the appearance of progressively enlarging nodular swellings of the adenoepidermal reticular network; b) continued swelling of this network with the concentration of fibroblasts in the area of swelling; c) formation of new fibrils from the swelling which created a dense structured network about the fibroblasts; and d) the aggregation of the concentrated fibroblasts to form small

nodules. Interbreeding of the normal animals from the first litter resulted in the production of 19 tumor-bearing newts. Autotransplants were successful.

Reports of skin tumors in anurans have been more numerous and of greater variety than those reported in the urodeles. The first report of an anuran tumor was by Eberth (71) in 1868. The author observed multiple tumors on the dorsal and ventral surfaces of a frog (*Rana* sp.). The tumors were composed of stratified squamous epithelium arranged to form tubules and acini that resembled normal skin glands. The author suggested that a parasite might possibly have had some causal relationship to this tumor since nematodes and trematodes are frequently present in the skin glands of the frog. Transplantation experiments were conducted but failed.

An adenocarcinoma of the skin of a frog (*Rana* sp.) was reported by Murray in 1908 (72). The tumor was hemispherical and located on the inner aspect of the thigh of an adult male. The neoplastic cells formed irregular tubules and acini that were separated by connective tissue septa.

Pavlovsky (73) reported two cases of an adenoma and one case of an adenocarcinoma in a frog (*Rana* sp.) in 1912. All of the lesions had one common morphological feature, that of tubules extending to the surface. The tubules were lined by tall columnar epithelium of the embryonal type, cells not normally present in frog's skin. Invasion was observed, and the lesion was interpreted as an adenocarcinoma.

Adenomas, 24 in number, were reported on the trunk and extremities of a *Rana* species by Pentimalli in 1914 (74). The largest of these

lesions was the size of a pea. The tumors were heavily vascularized, some showed ulceration, and all were histologically adenomas. Papillary proliferations sometimes extended into cysts which were present in the neoplastic tissue. No metastases were observed; attempts to transplant the lesion failed.

A lesion similar to those reported by Murray was described by Secher (75) in one specimen of *Rana esculenta*. This was a cauliflower-like mass, 1 cm in diameter, located on the outer surface of the thigh. The tumored epithelium was arranged in solid sheets or acini, some of which were cystic. Metastasis was not evident, and there was no evidence of parasites.

In 1923, Masson and Schwartz (76) described three glandular neoplasms found on the flank and abdomen of a frog, *Rana esculenta*. The pale pink, spherical tumors ranged from 5 to 15 mm in diameter and involved the epidermis only. Tubular spaces were bordered by neoplastic columnar epithelial cells and were supported by cuboidal cells. The tubules were often cystically dilated; sometimes these cells formed solid strands. The stroma was infiltrated by inflammatory and neoplastic cells which penetrated into the endothelium of the lymph sac. The author was undecided as to whether metastases had occurred. Transplants failed to grow.

Two cutaneous tumors were found between and around the eyes of a bullfrog, *Rana catesbiana*, by Duany in 1929 (77). Another lesion which approximated the size of the frog's head was found slightly posterior to the first two. The neoplastic epithelial cells formed tubules that resembled normal skin glands. There was no tissue invasion. The author

was unsure as to whether one of the smaller tumors was a primary tumor, or a metastasis or extension from the large lesion. The tumors were thought to be adenocarcinomas.

Pirlot and Welsch (78) in 1934 found 17 cases of cutaneous adenomas among 1800 frogs, *Rana fusca*. The tumors varied in size and number, and consisted of stratified columnar epithelial cells lining irregular acini. Mitotic figures were numerous, but there was no evidence of metastasis.

Llambes and Garcia (79), in 1949, described adenoepitheliomas of a bullfrog. The lesions were found on the leg, abdomen, and between the eyes. Homotransplantation of a tumor fragment to the anterior chamber of the eye resulted in rapid growth of the tissue in the new host.

Stolk (80), in 1947, investigated a single instance of multiple benign adenomas scattered over the skin of a specimen of *Rana arvalis* (Nelson). The tumors varied in size from 1 to 5 mm, and consisted of stratified squamous epithelium arranged as tubules, acini, or solid sheets supported by a delicate stroma. The nuclei of the cells were large, vesicular, and polymorphic, and the cytoplasm contained inclusions of unknown significance which were solitary or in small groups. No infiltration or metastasis was observed.

In 1958, Stolk also reported two other tumors in anurans: a guanophoroma of the skin of *Hyla arborea meridionalis* (81) and a xanthophoroma in *Hyla arborea* (82). The guanophoroma was located between the eyes and tympanic membrane. Histologic examination revealed spindle-shaped cells containing guanine crystals. This lesion was

poorly vascularized and infiltration apparently had not occurred. The orange colored xanthophoroma was located behind the tympanic membrane. This tumor contained large epithelial cells and single and multinucleated giant cells. The cells possessed orange-yellow cytoplasmic pigment granules. Unlike the guanophoroma, this tumor was heavily vascularized with some evidence of hemorrhage and necrosis. The author was unsure as to whether this was a true neoplasm or a granulomatous reaction to injury.

An erythrophoroma of the skin in a *Dendrobates typographicus* was also reported by Stolk (83). The tumor was located on the trunk and was red in color. Histologically, there were fusiform, vacuolated cells containing small, red, cytoplasmic granules, similar to those observed in normal erythrophores.

In 1961, Stolk (84) observed eight toads, *Bufo bufo japonicus*, with dorsal nodular swellings. These tumors consisted of fusiform fibroblasts surrounded by interlacing bundles of connective tissue fibers. The author noted the similarity of these tumors to a fibroma reported in the urodele *Triturus taeniatus* (70).

An adult female frog, *Xenopus laevis* D., that had been used several times for pregnancy testing, developed a melanoma involving one nostril (85). Microscopically, the tumor was papillary and composed of neoplastic melanocytes. The author felt the cells were not invasive.

Skin Tumors in Reptilia:

In 1938, Lucké (86) reported multiple large papillomas on the skin and eyes of three edible green turtles, *Chelonia mydas*. Bilateral

growth in one case had involved both eyes, resulting in blindness. The lesions were hemispherical, sessile with a pedunculated base, and measured from a few millimeters up to 20 cm in greatest dimension. The surfaces were rough, warty, and occasionally ulcerated. Histologically, the tumors were typical epidermal papillomas consisting of a connective tissue stroma covered by several layers of keratinized squamous epithelium.

Smith and Coates (1938) (39) described multiple papillomatous growths on six green turtles. The tumors were of two histological types, and both lesions were occasionally present on the same specimen. One type was a typical epidermal papilloma; the other was a smooth-surfaced growth which, on histological section, revealed interlacing bands of connective tissue of varying density covered by a thin cap of epithelium. The investigators classified the second lesion as a fibroma. In a subsequent manuscript (87) it was reported that over half of the 250 tumors present on the six specimens contained ova of the trematode, *Distomum constrictum*. No importance was attributed to the presence of these ova in the primary induction of the lesions. The authors noted the similarity of this condition to papillomatous and malignant urinary bilharziasis of humans.

Nigrelli (88) reported similar tumors on the green turtle and noted large numbers of leeches, *Ozobranchus branchiatus*, attached to the tumor surfaces. Those areas where firm attachment was made were characterized by thickened, keratinized epithelium with a highly vascularized stroma. Nigrelli stated that the etiology of these tumors is unknown, but suggested that the ectoparasitic annelids may play some

role in their development, perhaps as a vector for viruses or other parasites that may induce the papillomas. He also suggested that the hirudin secreted by the leeches may have a direct or indirect effect on the growth of the lesion.

Two musk turtles, *Sternothaerus odoratus*, were examined by Schlumberger and Lucké (41) and found to have tumorous growths on the flippers or tails, or both. The tumors were generally similar to the common human wart. There was a loose, well vascularized connective tissue core covered by multiple layers of stratified squamous epithelium showing hyperkeratosis and parakeratosis. There was no invasion of the stroma by the epithelium. Attempts to transplant the tumor in homologous and heterologous reptiles failed.

One instance of a skin tumor in Crocodilia has been reported (89) by Hansemann (1905). The lesion was similar to the common human wart and occurred on a crocodile (species not given) in the Berlin Aquarium.

There are a few reports in the literature of tumors in lizards (Sauria); most were tumors of the surface epithelium. The first such tumor was reported by Koch (90) in 1904. Several papillomas were observed on the frontal, parietal, and occipital regions of the head, as well as on the thorax, of a male lizard, *Lacerta agilis*. Grossly, the lesions were warty in appearance; histologically, they were typical papillomas. Heller (91) observed keratinized masses in the inguinal regions of several lizards, *Lacerta muralis fiumensis*. The lesions were similar to keratinizing condylomata acuminata in man. Plehn (92), in 1911, observed similar papillomatous lesions of various sizes occurring on several parts of the body in another species of lizard,

Lacerta viridis. There was no evidence of malignancy in any of these cases.

A squamous cell carcinoma arising from the skin of the forefoot of a tiga, *Tupinambis teguixin*, was reported by Schwartz (93) in 1923. The spherical lesion measured 3 cm in diameter and had destroyed the metacarpals and phalanges of the fourth and fifth digits. The tumor parenchyma consisted of neoplastic epithelial cells that showed suggestions of keratinization and "pearl" formation. A vascular stroma supported the groups of tumor cells, which appeared to infiltrate the adjacent muscle. There was no evidence of metastasis.

In snakes there are only a few reports of neoplasms, all of which were melanomas. In 1946, Ball (94) described melanomas on two pine snakes, *Pituophis melanoleucus*, a male and a female housed in the same cage. The female showed evidence of dark, rapidly growing tumors on the tail three years after capture. The tail was amputated and two similar lesions appeared two years after the appearance of the first lesion; there was also a fusiform swelling anterior to the cloaca. At autopsy, it was found that the tumor had infiltrated the body cavity, and there was metastasis to the liver. The tumor cells were spindle-shaped with abundant cytoplasm containing fine brown pigment granules. The cells formed interlacing bundles and palisades. The male snake developed a similar lesion, located on the labial fold, six years after capture. The animal was sacrificed, and the lesion was identified as a melanosarcoma.

Two instances of melanomas in reticulated pythons, *Python reticulatus*, were observed by Schlumberger and Lucké (41). The first

appeared on the lateral surface of a 50- to 60-year-old female python, grew rapidly, and was lobulated and ulcerated. At autopsy there was invasion of surrounding tissue structures. In the second specimen there were two tumors on a 20-ft female. The tumors occurred on the jaw and body and were histologically similar to the melanomas of pine snakes described by Ball (94), except that the tumor of the jaw was almost without pigment. Both tumors were interpreted as benign melanomas.

In seven specimens of the lizard, *Lacerta agillis* L., nodules measuring 0.5 cm in diameter were scattered irregularly over the skin (95). Histologically, there was marked hyperkeratosis, grading into epidermoid carcinoma, with pearl formation. The lizards had been kept in cages with only minimal amounts of light; other individuals of the same species exposed to direct sunlight, and on the same diet, developed no lesions.

Stolk (96), in 1957, reported a melanoma in an adult viper snake, *Vipera berus*. The tumor was located on the dorsal skin, midway between the head and tail, and was a firm, dark-colored, lobulated, irregular mass with large and small nodules over its surface. The cut surface displayed irregularly arranged pigmented and non-pigmented areas. Microscopically, spindle-shaped cells with abundant cytoplasm were arranged in intersecting bundles. The nuclei were fusiform and formed palisades. Three types of tumor cells were described with distinct transitions between pigmented and nonpigmented cells. Infiltration of adjacent tissue was observed.

MATERIALS AND METHODS

1. NATURAL HISTORY STUDIES

a. Collection:

Fish in this study were collected in Bellingham Bay, Bellingham, Washington. Specimens were collected by means of a shrimp "try" net (0.5 inch stretch mesh in the bunt) or an otter trawl (1.5 inch stretch mesh in the bunt) operated from the fishing vessel, *Hydah*. A 15-foot beach seine (mesh size, 0.25 inch) was used to collect early postmetamorphosis starry flounders along the shore of the southwest side of the Nooksack Estuary.

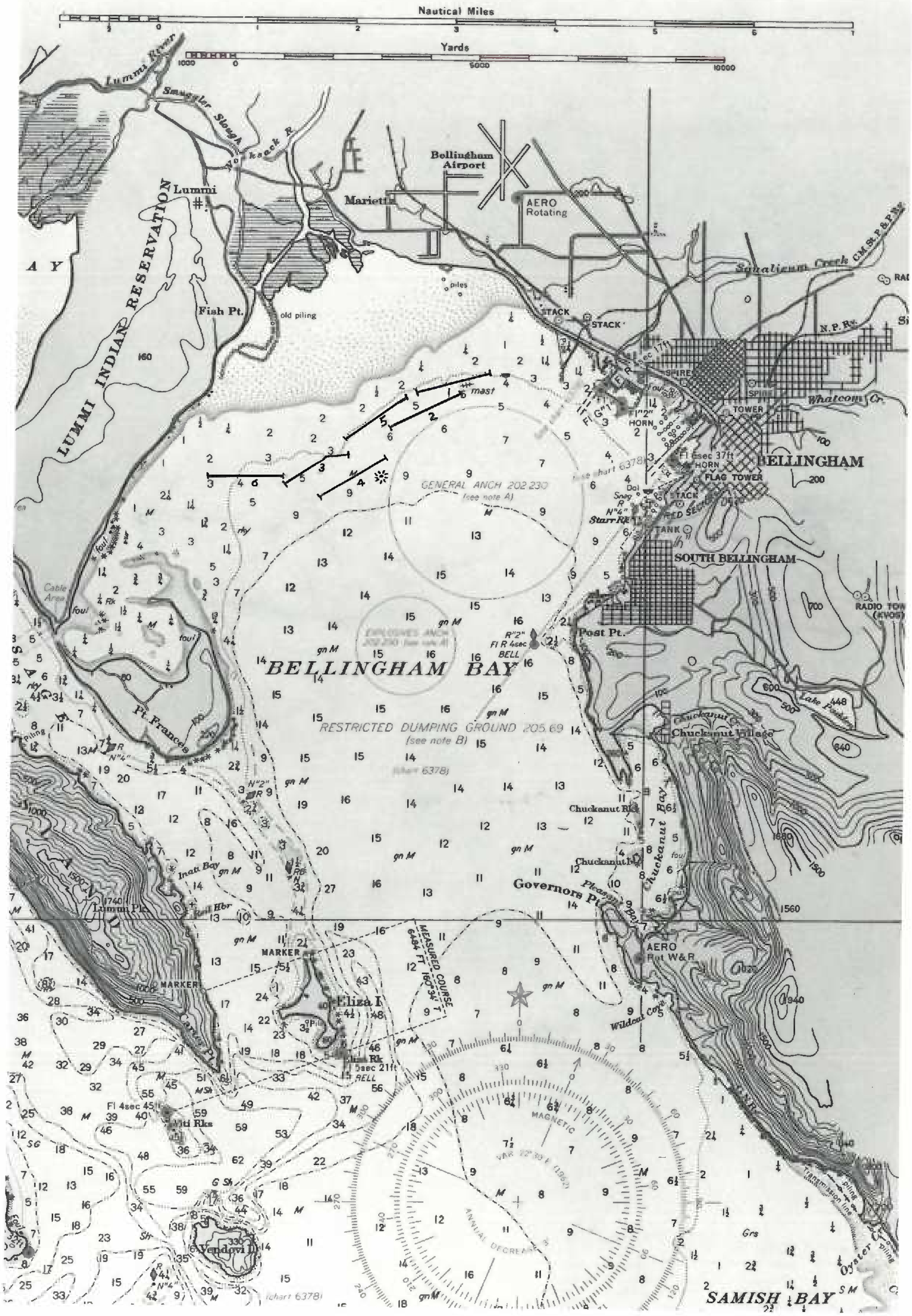
Definite sampling stations were established during the first collection in October, 1966; these stations were sampled at monthly intervals thereafter for one year (Chart 1). The standardized collection procedures consisted of six 15-min tows of approximately 1,600 yards each at depths of 4 to 7 fathoms. Water depths were obtained from soundings at mean low water on Coast and Geodetic Survey maps and by actual measurement with a bathythermograph. Temperatures of the air, surface water, and bottom water were also measured.

The composition of the bottom of the Bay consisted of variable proportions of sand and mud, which were either uncovered or covered with sea shells or decaying wood (never both). Collecting stations were reproducibly located by reference to the topography of the shore and to the type of bottom material characteristic of that collection site. The shoreline varied from rock to sandy beaches and was bordered by many domestic dwellings and commercial industries.

Chart 1:

Standardized sampling stations in Bellingham Bay, Washington . A photograph of Coast and Geodetic Survey Chart No. 6380.

* indicates the site where temperatures were measured and plankton tows were performed.


Nautical Miles
Yards
Lummi River
Smuggler Slough
Nooksack R.
Bellingham Airport
AERO Rotating
Lummi
Marietta
LUMMI INDIAN RESERVATION
Fish Pt.
old piling
piles
STACK
Squalicum Creek
N.P. Ry.
Whatcom Cr.
SPIRE
TOWER
HORN
FLAG TOWER
BELLINGHAM
Starr Rk
TANK
SOUTH BELLINGHAM
GENERAL ANCH 202.230
EXPLOSIVES ANCH 202.230
Post Pt.
RADIO TOWER (KVOS)
Cable Area
foul
BELLINGHAM BAY
RESTRICTED DUMPING GROUND 205.69
(see note B)
Pt. Frances
Lake Padden
Chuckanut Cr.
Chuckanut Village
Chuckanut Bay
Chuckanut Rk
Governors Pt.
Pleasant Bay
Inati Bay
Reil Hbr
Lummi Pk.
MARKER
MEASURED COURSE
Eliza I.
AERO Rot W&R
Wildcat Cove
MAGNETIC
ANNUAL DECREASE
Viti Rks
Vendovi I.
Transmission line
Oyster Cr.
SAMISH BAY

After collection the fish were placed in live boxes containing circulating sea water, sorted by species, measured, and examined for evidence of skin lesions. Some of the normal and tumor-bearing fish were placed in separate live boxes and transported to the Friday Harbor Laboratories of the University of Washington, where they were maintained in aquaria and fed fresh minced fish and clams.

Normal fish and fish with tumors, maintained separately or together, were observed for periods of 6 months to 1 year at Friday Harbor, and in a 25-gal "Instant Ocean Culture System" (Aquarium Systems, Inc., Wickliffe, Ohio), located at the University of Oregon Medical School.

The eggs of gravid starry flounders were stripped into clean finger bowls containing sea water. A few drops of sperm from mature, ripe male starry flounders were placed in another finger bowl containing 5-ml of sea water. The eggs were artificially fertilized by mixing the two. Embryological and larval development was observed for 6 days, at which time the yolk sac was depleted and the larvae died, apparently from starvation.

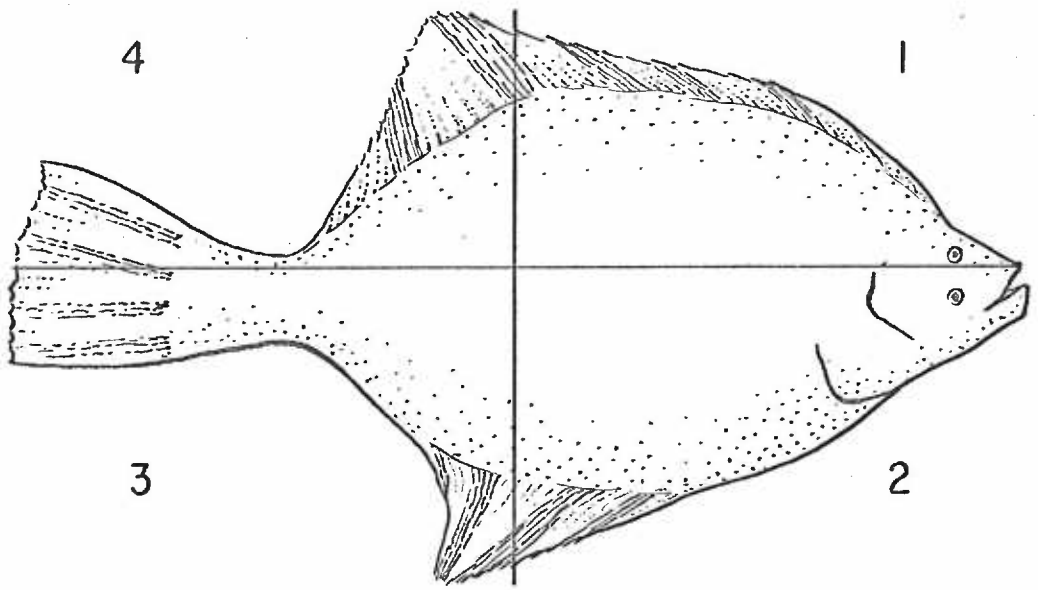
b. Processing of Tumor Fish:

The locations of the tumors on the fish were tabulated according to quadrants as indicated on Chart 2. Fish possessing tumors were measured (total length and standard length), sexed, and grouped according to age by counting the number of annual growth rings on the interopercular and subopercular bones and on the auditory ossicles. The designation age group 0 indicates no annual growth ring and an age of 0 to 1 year; age group I indicates one annual growth ring and an age of

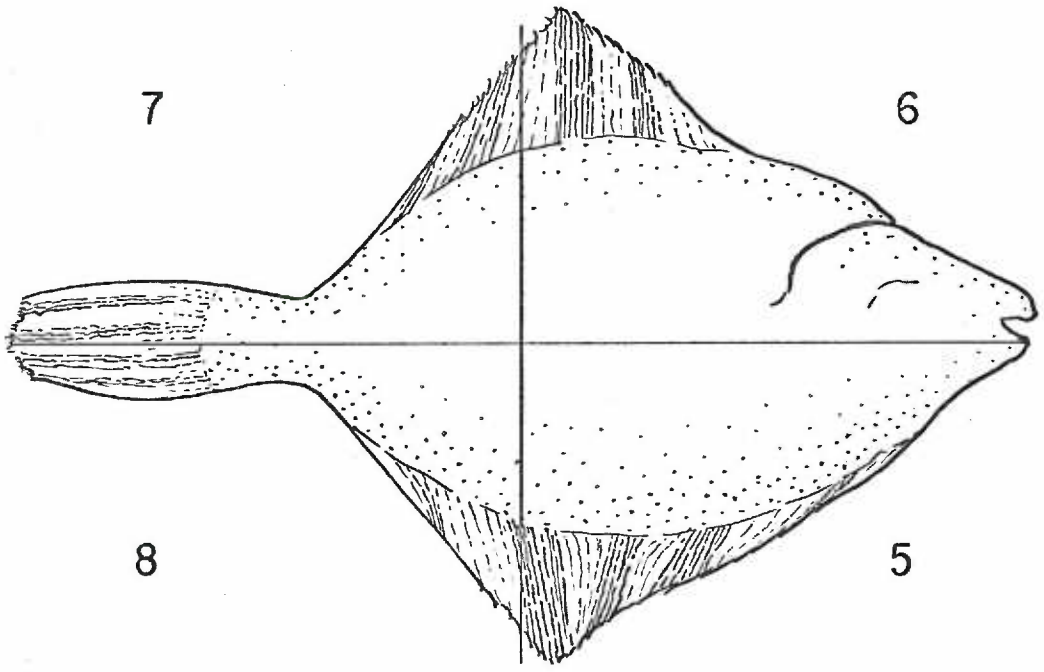
Chart 2:

Quadrants used to tabulate the location of tumors on the pigmented and nonpigmented sides of starry flounder and the English sole. The quadrants were established by drawing a line from the tip of the snout to a midpoint at the tip of the tail. This line, when divided in half by a line running perpendicular to the first, establishes the four quadrants on each side.

PIGMENTED (EYE) SIDE



NONPIGMENTED SIDE



1 to 2 years; age group II indicates two annual growth rings and an age of 2 to 3 years, *etc.*

c. Storage in the Field:

Tumor tissue for virological studies was removed by means of sterile instruments, sealed in sterile vials, and placed in a biological transport container BT-3 (Union Carbide Corp., Oak, California) which was precooled with liquid nitrogen. Tissue held for periods longer than 3 days was transferred to a LR-35-9 liquid nitrogen refrigerator (Union Carbide Corp.) at -190 C.

Normal organs and tumor for tissue culture were removed under sterile conditions, minced, and transported to the laboratory, at 4 C, in medium 199 (97) containing 10% fetal calf serum (Microbiological Associates, Albany, California), 100 units of penicillin (Charles Pfizer and Co., Inc., New York, New York), and 200 µg of streptomycin (E. R. Squibb and Sons, New York, New York).

2. HISTOPATHOLOGICAL STUDIES

a. Light Microscopy:

Tissue for light microscopy was fixed in Bouin's fluid or 10% formalin and embedded in paraffin. Five-µ sections were cut on a model "820" rotary microtome (A. O. Spencer). These were stained by one or all of the following methods: hematoxylin and eosin, Masson's trichrome, periodic acid Schiff (PAS), Gridley's reticulum, Feulgen, Schorr's, and Giemsa's stains (98).

b. Electron Microscopy:

Tissue for electron microscopy was fixed in Lentz's fixative (99) or 1.33% OsO_4 buffered with S-collidine (100), embedded in Swiss araldite (101), and polymerized at 60 C in an oven for 7 to 10 days. Thick (1 μ) and thin (50-100 mμ) sections were cut on an LKB Ultrotome (LKB Instruments, Inc., Washington, D. C.) equipped with a diamond or glass knife and mounted on 100- to 150-mesh copper grids coated with parlodion (Mallinckrodt Chemical Works, New York, New York) and carbon. The thick sections were stained with Richardson stain (102), mounted in Histoclad (Clay-Adams, Inc., New York, New York), and viewed with a light microscope. The thin sections were triple stained, first with lead citrate (103) for 5 min, then with saturated uranyl acetate for 1 min, and then once again with lead citrate for 8 min. Sections were viewed by means of an RCA EMU 3-G electron microscope operated at 50 KV or a Philips EM 200 electron microscope operated at 60 KV.

3. VIROLOGICAL STUDIES:

ATTEMPTS TO ISOLATE VIRAL AGENTS AND TEST FOR BIOLOGICAL ACTIVITY

a. Homogenization of Tumor and Normal Tissues:

Small amounts of tumor or normal tissue were placed in pre-weighed sterile petri dishes and weighed. The tissue was then transferred to a container and homogenized with 1 to 2 ml of buffer in one of the three following ways: (1) by means of a cold mortar and pestle containing sterile alundum and 0.01 *M* phosphate-buffered saline (PBS), pH 7.0; (2) by means of an Omni-mixer (Ivan Sorvall, Inc., Newark, Conn.) cooled to 4 C, employing 0.01 *M* tris(hydroxymethyl)-aminomethane buffer (tris),

(pH 7.5), operated at maximum speeds for 15 sec and cooled for 15 sec through eight cycles; (3) or in a Virtis mixer (The Virtis Co., Inc., Garner, New York) containing TES buffer (0.01 *M* tris, 0.001 *M* ethylenediaminetetraacetic acid, disodium salt [EDTA], and 0.05 *M* NaCl), pH 7.5, and operated at high speeds for 2 min with interruptions for cooling. Sonication at 20 kc for four 15-sec pulses with interruptions for cooling was sometimes used in conjunction with the homogenization. After homogenizing, sufficient buffer was added to the homogenate to make a final 20% (W/V) suspension of tissue.

b. Extraction of Tissues:

Three extraction procedures were used: a modified Noyes' (104) genetron procedure, the sucrose acetone extraction method described by Clarke and Casals (105), and the nucleic acid extraction method of Ito (106). The genetron extraction method used was as follows: one-half volume of redistilled Freon 113 (Matheson Co., Newark, California) was added to the tissue to be extracted. Eight 15-sec cycles of homogenization at maximum speeds and cooling in an Omni-Mixer were used. The homogenate was then centrifuged at 2000 rpm (type SB centrifuge, International Equipment Co., Boston, Mass.) for 5 min at 4 C in 15-ml conical glass centrifuge tubes, and the aqueous layers were removed after centrifugation and saved. The middle and bottom layers were washed by homogenizing them in one-half volume of 0.01 *M* PBS, pH 7.0, or 0.01 *M* tris buffer, pH 7.5, and centrifuging the homogenate at 2000 rpm. The aqueous layer from this procedure was combined with the first aqueous layer, and a second cycle of extractions was

done, using the same procedure. After completion of the second cycle, the aqueous layers were combined and placed in 5-ml cellulose nitrate tubes (Beckman Instruments, Palo Alto, California) and centrifuged at 166,647 X *g* max. in a SW 39L rotor on a Beckman model L ultracentrifuge (Beckman Instruments, Inc., Palo Alto, California) for 3.5 hr at 4 C. The tubes were examined for the presence of a precipitate, which was saved. The supernatant was removed, made up to 5 ml with PBS or tris buffer (mentioned above) and centrifuged at 166,647 X *g* max. for 9 hr at 4 C. After this, the supernatant was discarded. The two precipitates were resuspended in pH 7.5 tris buffer, 0.01 *M*, stained with 2% phosphotungstic acid (PTA), pH 6.0, and viewed with the electron microscope or subjected to other tests.

For a description of the sucrose-acetone extraction procedure, refer to reference (105).

Attempts to extract "infectious" nucleic acid from tumor tissue were done with the help of Dr. Yohei Ito, who has successfully transmitted Shope papilloma by this method (106). The procedure used was his modification of "cold" and "hot" phenol methods.

c. Isolation Procedures:

1) Differential centrifugation procedures used in attempting to isolate viruses from tumor tissue varied, but in general included a clarification of the tissue homogenates at speeds of 2000 to 5000 rpm for 5 to 10 min. The precipitate was diluted with distilled water, stained with PTA, and viewed with the electron microscope. The supernatant was spun at 166,647 X *g* max. for 2 hr at 4 C in the SW 39L rotor.

The supernatant was aspirated, and the precipitate was resuspended in 1 ml of 0.01 *M* PBS, pH 7.0, 0.01 *M* tris, pH 7.5, or TES, pH 7.5, by sonication (20 kc). The above procedure of clarification and precipitation was repeated 3 times. The resuspended precipitate was either inoculated into cell cultures, stained with heavy metal for electron microscopy, or subjected to density gradient centrifugation.

Three methods for making density gradients were used in the isolation procedures.

2-a) Sucrose gradients were prepared by placing, in order, 8 ml of 61, 53, 45, 37, and 21% sucrose solutions (Merck and Co., Rahway, New Jersey) in 5-ml cellulose nitrate tubes (107). The sucrose was made in 0.3% NaCl in distilled water. These gradients were placed in a 4 C refrigerator overnight. This allowed the gradient to become continuous by diffusion. The next morning, 1 ml of sample was placed over the gradients and centrifuged at 166,647 X *g* max. in a SW 39L rotor for 3 to 16 hr at 4 C.

2-b) Alternatively, sucrose gradients were prepared in a custom-made gradient-making device. Two solutions of sucrose, one light (25% in 0.1 *M* tris buffer, pH 8.0) and the other heavy (40% in 0.1 *M* tris buffer, pH 8.0) flowed into a common chamber where they were mixed and delivered as a preformed gradient into a 5-ml cellulose nitrate tube. These gradients were less dense than those mentioned in 2-a above, and a rate zonal density gradient procedure was employed in an attempt to isolate virus particles from tumor tissue. Specimens were spun for either 45 min or 4 hr at 166,647 X *g* max. at 4 C.

3) Gradients were also made with 38.5% potassium citrate (reagent, crystals, Matheson, Coleman, and Bell, Los Angeles, California) in distilled water. The potassium citrate (4 ml) was placed in 5-ml cellulose nitrate tubes which were then placed in a -20 C freezer overnight. The frozen material was then allowed to thaw at room temperature, thus forming a linear gradient. The density of the 38.5% solution was approximately 1.30. One milliliter of material was placed over this gradient and centrifuged for 3 hr at 120,000 X *g* max.

Bands in the gradient tubes after spinning were observed by Tyndall illumination and removed by puncturing the side of the tube and aspirating with a syringe and 27-ga needle, or by puncturing the bottom of the tube with a 27-ga needle and collecting fractions in a dropwise manner (12-drop fractions).

Band material to be placed on cell cultures, injected into experimental animals, or viewed by the electron microscope, were exhaustively dialyzed against 0.01 *M* PBS, pH 7.0. The dialysate was then placed in tubes and spun at 166,647 X *g* max. at 4 C for 2 hr to remove particulate matter. The particulate matter was resuspended in a small amount of 0.01 *M* PBS, pH 7.0, inoculated into cell cultures, injected into experimental animals, or diluted 1:1 with PTA (pH 6) and viewed with the aid of the electron microscope, or stored in a Revco deep freeze (Revco, Inc., Deerfield, Mich.) at -80 C.

d. Application of Test Material to Indicator Systems:

Each of the following were tested for the presence of viral agents: untreated homogenates, differential centrifugation precipitates and supernatants, and dialyzed density gradient bands.

d-1) Cell culture studies--

Small volumes of these materials (0.5 to 2 ml) were placed on grunt fin (108), rainbow trout gonad (109), and fathead minnow (110) cell culture lines for 10 min. The cells were then covered with medium M199 containing 10% agamma calf serum (111). The cultures were observed for cytopathogenic effect (CPE) and any other abnormal changes. Monolayers of the same cells on coverslips in Leighton tubes were also inoculated with the suspected material by the same method. Coverslips were removed at daily intervals, stained with Giemsa, and observed for any abnormalities (*e.g.*, inclusion bodies or CPE). The growth media were removed and stored at -80 C. At a later date this stored material was removed from the deep freeze, thawed at room temperature, and spun at 166,647 X *g* max. for 2 hr. The precipitate was stained with PTA and examined for virus particles by the electron microscope.

d-2) Hemagglutination procedure--

Domestic white goose erythrocytes, *Anser cinereus* (Colorado Serum Co., Denver, Colorado) were prepared and stored according to the procedure of Clark and Casals (105). The optical density (OD) per unit volume was measured in a Coleman Junior Photoelectric Spectrophotometer. Small samples of the cell suspension were diluted 1:40 in 0.9% NaCl and the OD was measured at 490 mμ, using 10-mm tubes. By using the total volume of the cell suspension and the OD, the suspension of cells can be diluted to any desired concentration (final volume = initial volume X observed OD/desired OD). Cell suspensions were made up weekly and stored at 5 C when not in use.

The hemagglutination procedure, which utilized the sucrose acetate extracted antigen and the specially prepared goose cells, was conducted exactly as described by Clark and Casals (105). Dilutions of each hemagglutinin preparation were tested against goose erythrocytes at pH 5.75, 6.0, 6.2, 6.4, 6.6, 6.8, 7.0, 7.2, 7.4, and 7.6. Three identical sets were used in each case; one set was incubated at 4 C, the second set was incubated at 20 C, and the third set was incubated at 37 C. Crude antigens, *i.e.* tissue not treated by the above extraction, were also tested using the above procedure. Cell controls were used to insure that proper settling had occurred.

e. Transplantation Procedures:

More than 200 pleuronectids (starry flounders, English sole, and flathead sole), from Bellingham Bay and the East Sound, Orcas Island, Washington, were used in attempts to transmit the disease autologously, homologously, or heterologously. Tumor transmission was attempted by four methods: 1) cell-free filtrates, prepared from the final product of differential centrifugation (previously described) were used. The final precipitate was resuspended in sterile 0.01 *M* PBS, pH 7.0, and injected (0.05 ml) intradermally or intraperitoneally, or placed upon the scarified epidermis. 2) Tumor fragments, 1 x 1 x 2 mm, were placed under the skin or into the anterior chamber of the eye. 3) 0.1 ml cell suspensions of tumor tissue, in 0.01 *M* PBS, pH 7.0, were injected intraperitoneally, and 4) by means of "infectious" nucleic acid prepared by the method of Ito (previously described). For inoculation of "infectious nucleic acid", the epidermal surface of the recipient fish was blotted dry with sterile gauze; a sterile multineedled device was dipped in the

nucleic acid preparation and plunged through the epidermis and dermis and into the musculature of the fish. Injection of cell-free filtrates was performed as follows: each fish was injected in the fin web, the lateral line, and predetermined sites of the body surface. All injections were into the pigmented side.

RESULTS

NATURAL HISTORY

All fishes and invertebrates collected in Bellingham Bay were examined for tumors and other surface abnormalities. Ten species of Heterosomata, including representatives of the families Pleuronectidae (flounders) and Bothidae (sanddabs) were represented; tumors were observed only on pleuronectids (Table 2). Of the 5,240 pleuronectids collected, a total of 1,977 English sole (*Parophrys vetulus*) were examined, and 95 or 4.8 per cent had skin tumors. Forty-two, or 1.5 per cent, of the 2,894 starry flounders (*Platichthys stellatus*) collected from the *M. V. Hydah* had similar skin tumors, whereas 28 (or 54%) of the 52 starry flounders collected by beach seine were tumorous. The number of starry flounders collected by beach seine and from the fishing vessel therefore totalled 2,946, of which 71 (or 2.4%) had tumors.

Fish with solitary and multiple tumors were observed in both of the above species. There was, however, a tendency for the number of tumors per fish to vary inversely with the age of the fish. English sole of age group 0 had as many as 22 tumors, for a yearly average of 4.06 tumors per tumorous fish. English sole of age group I had as many as 17 tumors on a single specimen for an annual average of 2.54 tumors per tumorous fish. No tumors were found on fish in age group II or older.

TABLE 2

Incidence of Skin Tumors Among Pleuronectidae and Bothidae
From Bellingham Bay, Washington

12 Monthly Collections
October, 1966 to October, 1967

Scientific Name	Common Name	No. Tumorous Fish / Total No. Fish	Per Cent Tumorous Fish
Parophrys vetulus	English sole	95/1977	4.80
Platichthys stellatus	Starry flounder	71/2946	2.41
Psettichthys melanostictus	Sand sole	2/61	3.28
Isopsetta isolepsis	Butter sole	0/203	0
Citharichthys sordidus	Mottled sanddab	0/22	0
Lepidopsetta bilineata	Rock sole	0/21	0
Glyptocephalus zachirus	Rex sole	0/4	0
Hippoglossoides elassodon	Flathead sole	0/3	0
Microstomus pacificus	Dover sole	0/2	0
Atheresthes stomias	Arrowtooth flounder	0/1	0

One right-handed (dextral*) starry flounder, age group 0, which was collected in 4 to 5 fathoms of water from the *M. V. Hydah*, had three tumors located on its body. Dextral starry flounders of age group I had 1 to 18 tumors and a yearly average of 2.5 tumors per tumorous fish. Only solitary tumors were found on dextral starry flounders in age group II. No tumors were found on fish older than age group II.

Only two sinistral tumor-bearing starry flounders of age group 0 were collected from the *M. V. Hydah*. One of these had 31 tumors, and the other fish had no tumors, resulting in an annual average of 15.5 tumors per fish. Tumor-bearing sinistral starry flounders of age group I had 1 to 7 tumors per fish for a yearly average of 2.1 tumors per tumorous fish. Only solitary skin tumors were observed on sinistral starry flounder of age groups II and III; no tumors were found on older fish.

Tumor-bearing English sole of age group 0 were found predominantly in collections 1 through 5, corresponding to the months of October through February (Table 3). A single tumor-bearing fish of age group 0 was observed in the May collection; this specimen represented the only instance in which a tumor was observed between February and August on

---

*Starry flounders are unusual among pleuronectids in that after metamorphosis the fish may be either dextral with the right side uppermost in relation to the substrate, or sinistral, with the left side uppermost in relation to the substrate. In either case, the uppermost side possesses both eyes and most of the pigment. Dextral individuals are sometimes referred to as being "right-handed" and sinistral individuals "left-handed". The proportion of dextral and sinistral individuals varies in collections from different geographical locations.

TABLE 3

ENGLISH SOLE: TUMOR INCIDENCE BY AGE GROUP AND COLLECTION DATE

Collection Number	Date	Number of Tumorous Fish: Age Group 0 (0-1 yr)	Age Group I (1-2 yr)	Age Group II (2-3 yr)	Age Group III (3-4 yr)	Number Tumor Fish Age Determined	Total Fish With Tumors	Total Fish
1	10-3-66	15*	--	--	--	9	15	216
2	11-4-66	4	3	--	--	7	7	41
3	12-21-66	5	4	--	--	9	9	362
4	1-13-67	10	6	--	--	16	16	411
5	2-7-67	3	20	--	--	23	23	332
6	3-3-67	--	--	--	--	--	--	--
7	3-31-67	--	2	--	--	2	2	7
8	5-5-67	1	2	--	--	3	3	81
9	6-2-67	--	2	--	--	2	2	91
10	7-18-67	--	5	--	--	5	5	183
11	8-14-67	--	4	--	--	4	4	132
12	9-14-67	9	--	--	--	9	9	121
TOTAL		47	48	--	--	89	95	1,977

Per cent tumor incidence of total fish with tumor = 4.81.
Per cent tumor incidence of age determined tumor fish = 4.50.

*Six of the 15 fish died. Their ages were not determined by reading growth rings (annuli) on their ear ossicles. These six fish were age grouped according to their total and standard lengths.

English sole of age group 0*. The incidence of tumors in group 0 fish rose sharply in the month of September. Tumorous English sole of age group I were found throughout the year. The incidence of tumors in the English sole increased from collection 2 through collection number 5 (November through February) and decreased thereafter. No group I fish were found when the incidence of tumors in group 0 fish was highest (*i.e.*, collections 1 and 12) and the highest incidence of tumors in age group I occurred when the numbers of group 0 tumorous fish had declined markedly. Tumors in English sole older than age group I were not observed.

Sinistral starry flounders of age group 0 were seldom collected from the fishing vessel (Table 4), and only two of these were tumor bearing. However, beach seine collections during the months of July, August, and September established the fact that numerous starry flounders of age group 0 were present at several collecting sites in shallow (6 in. to 3 ft.) protected waters. Thirteen (or 25%) of 52 starry flounders collected by beach seine in the Nooksack Estuary had tumors. Tumorous fish in age groups I, II, and III were collected between October and March.

The dextral starry flounders in the collections (Table 5) displayed essentially the same characteristics as the sinistral group as regards the distribution of fish by age group and collection date. Only one dextral tumor-bearing starry flounder of age group 0 was collected in 5

*Fish of age group 0 would be expected to be absent from bottom collections in spring and early summer because the fish of this age group are probably planktonic during this time interval.

TABLE 4

SINISTRAL STARRY FLOUNDER: TUMOR INCIDENCE BY AGE GROUP AND COLLECTION DATE, SINISTRAL SPECIMENS ONLY

Collection Number	Date	Number of Tumorous Fish: Age Group 0 (0-1 yr)	Age Group I (1-2 yr)	Age Group II 2-3 yr)	Age Group III (3-4 yr)	Number Tumor Fish Age Determined	Total Fish With Tumors	Total Fish
1	10-3-66	--	1	--	--	1	1	160
2	11-4-66	1	6	--	1	8	8	430
3	12-21-66	--	--	--	--	--	--	32
4	1-13-67	--	--	--	--	--	--	128
5	2-7-67	--	6	1	--	7	7	683
6	3-3-67	--	1	1	--	2	2	380
7	3-31-67	1	--	--	--	1	1	210
8	5-5-67	--	--	--	--	--	--	197
9	6-2-67	--	--	--	--	--	--	156
10	7-18-67	--	--	--	--	--	--	233
11	8-14-67	--	1	--	--	1	1	138
12	9-14-67	--	1	--	--	1	1	147
TOTAL		2	16	2	1	21	21	2,894

Per cent total tumor fish .726.
Per cent total tumor age determined 0.726.

TABLE 5

DEXTRAL STARRY FLOUNDER: TUMOR INCIDENCE BY AGE GROUP AND COLLECTING DATE, DEXTRAL SPECIMENS ONLY

Collection Number	Date	Number of Tumorous Fish: Age Group 0 (0-1 yr)	Age Group I (1-2 yr)	Age Group II (2-3 yr)	Age Group III (3-4 yr)	Number Tumor Fish Age Determined	Total Fish With Tumors	Total Fish
1	10-3-66	--	2*	--	--	2	4	160
2	11-4-66	--	4	--	--	4	4	430
3	12-21-66	--	--	--	--	--	--	32
4	1-13-67	--	2	--	--	2	2	128
5	2-7-67	--	1	--	--	1	1	683
6	3-3-67	1	3	1	--	5	5	380
7	3-31-67	--	1	2	--	3	3	210
8	5-5-67	--	1	1	--	2	2	197
9	6-2-67	--	--	--	--	--	--	156
10	7-18-67	--	--	--	--	--	--	233
11	8-14-67	--	--	--	--	--	--	138
12	9-14-67	--	--	--	--	--	--	147
TOTAL		1	16	4	--	19	21	2,894

Per cent tumor incidence, total tumor fish 0.723.
Per cent tumor incidence, age determined fish, 0.656.

*Two of the 4 fish died. Their age was not determined by reading growth rings (annuli) on their ear ossicles. These two fish were age grouped according to their total and standard lengths.

fathoms of water from the *M. V. Hydah*; however, 15 (28%) of a total collection of 52 fish of the same species and age group were collected in the Nooksack Estuary of Bellingham Bay by means of a beach seine during the months of July, August, and September. Tumor fish collected between October and May almost always were of age group I or older. No dextral starry flounders were collected from June through September from the *M. V. Hydah*.

All quadrants of the afflicted fish were apparently susceptible to tumor formation (Figs. 1-18). There was, however, a tendency for the tumors to occur more frequently on the pigmented side, rather than on the nonpigmented side of the fish. The chi-square test indicated that this tendency was significant for the English sole (Table 6). In this study, right and left-handed starry flounders collected from the fishing vessel showed no significant tendency for the lesions to occur on either side of the fish (Tables 7 and 8). Starry flounders collected by beach seine, however, showed a statistically significant tendency for more tumors to occur on the pigmented side than on the nonpigmented side (Table 9). In another study, the predominance of tumors on the pigmented side was also statistically significant (7).

GROSS MORPHOLOGY AND LIGHT MICROSCOPY

Normal Skin:

Histological slides of normal skin from starry flounders and English sole were stained with hematoxylin and eosin. In addition, epon embedded tissue sectioned at 1 μ was examined. The light microscopic morphology was identical for the two species and is illustrated in

TABLE 6

ENGLISH SOLE: TUMOR INCIDENCE ACCORDING TO AGE GROUP AND LOCATION ON SPECIMEN

Age Group	Number of Fish with Tumors	Number of Tumors on Pigmented Side	Number of Tumors on Nonpigmented Side	Number of Tumors on Both Sides	Total Number of Tumors	Range of Number of Tumors per Fish	Average Number of Tumors per Fish
0	47	110 (57.9%)	57 (30.0%)	23 (12.1%)	190	1-22	4.04
I	48	66 (49.3%)	43 (32.1%)	25 (18.7%)	134	1-17	2.81
II	0	0	0	0	0	0	0
III	0	0	0	0	0	0	0
TOTAL	95	176*	100*	48	324		

*χ^2 determination: in determining the significance of the difference in occurrence of the tumors on the pigmented versus nonpigmented side of the fish, all tumors that involved both sides of the fish were eliminated, since the initial site of origin was unknown.

$$\chi^2 = \sum \frac{(176-138)^2}{138} + \frac{(100-138)^2}{138} = \frac{(38)^2}{138} + \frac{(-38)^2}{138} = 20.92$$

χ^2 exceeds the significant level of 0.1% with one degree of freedom.

TABLE 7

STARRY FLOUNDER: TUMOR INCIDENCE ACCORDING TO AGE GROUP AND LOCATION ON SPECIMEN, DEXTRAL SPECIMENS ONLY

Age Group	Number of Fish with Tumors	Number of Tumors on Pigmented Side	Number of Tumors on Nonpigmented Side	Number of Tumors on Both Sides	Total Number of Tumors	Range of Number of Tumors per Fish	Average Number of Tumors per Fish
0	1	2 (66.6%)	0 (0.0%)	1 (33.3%)	3	1-3	3.0
I	17	18 (45.0%)	13 (32.5%)	9 (22.5%)	40	1-18	2.35
II	2	2 (50.0%)	0	2 (50.0%)	4	1	2.0
III	0	0	0	0	0	0	0
TOTAL	20	22*	13*	12	47		

*χ^2 determination: in determining the significance of the difference in occurrence of the tumors on the pigmented versus nonpigmented side of the fish, all tumors that involved both sides of the fish were eliminated, since the initial site of origin was unknown.

$$\chi^2 = \sum \frac{(22-17.5)^2}{17.5} + \frac{(13-17.5)^2}{17.5} = \frac{(4.5)^2}{17.5} + \frac{(4.5)^2}{17.5} = 2.30$$

χ^2 determinations: are less than the significant level of 5% with one degree of freedom.

TABLE 8

STARRY FLOUNDER: TUMOR INCIDENCE ACCORDING TO AGE GROUP AND LOCATION ON SPECIMEN, SINISTRAL SPECIMENS ONLY

Age Group	Number of Fish With Tumors	Number of Tumors on Pigmented Side	Number of Tumors on Nonpigmented Side	Number of Tumors on Both Sides	Total Number of Tumors	Range of Number of Tumors per Fish	Average Number of Tumors per Fish
0	2	19 (59.4%)	13 (40.6%)	0	32	1-31	16.0
I	16	11 (32.4%)	9 (26.5%)	14 (41.2%)	34	1-7	2.13
II	2	2 (100.0%)	0	0	2	0-1	1.0
III	1	0	0	1 (100.0%)	1	0-1	1.0
TOTAL	21	32	22	15	69		

χ^2 determination: in determining the significance of the difference in occurrence of the tumors on the pigmented versus nonpigmented side of the fish, all tumors that involved both sides of the fish were eliminated, since the initial site of origin was unknown.

$$\chi^2 = \sum \frac{(32-27)^2}{27} + \frac{(22-27)^2}{27} = \frac{(5)^2}{27} + \frac{(5)^2}{27} = 1.85$$

χ^2 determinations: are less than the significant level of 5% with one degree of freedom.

TABLE 9

STARRY FLOUNDER: OCCURRENCE OF SKIN TUMORS ON PIGMENTED VERSUS NONPIGMENTED SIDES, ALL SPECIMENS OF AGE GROUP 0, BOTH SINISTRAL AND DEXTRAL, COLLECTED BY BEACH SEINE

Number of Fish with Tumors	Number of Tumors on Pigmented Side	Number of Tumors on Nonpigmented Side	Number of Tumors on Both Sides	Total Number of Tumors	Range of Number of Tumors per Fish	Average Number of Tumors per Fish
28	42 (62.7%)	24 (35.8%)	1 (1.5%)	67	1-14	2.39

χ^2 determination: in determining the significance of the difference in occurrence of the tumors on the pigmented versus nonpigmented side of the fish, all tumors that involved both sides of the fish were eliminated, since the initial site of origin was unknown.

$$\chi^2 = \sum \frac{(42-33)^2}{33} + \frac{(24-33)^2}{33} = \frac{81}{33} + \frac{81}{33} = 2.45 + 2.45 \text{ or } 4.90.$$

χ^2 determination: exceeds the significant level of 5% with one degree of freedom.

Figures 19 to 30. The dermis and epidermis were separated by a distinct basement membrane. The epidermis consisted of a basal layer in which occasional mitoses were observed, an intermediate layer of cells possessing prominent "intercellular bridges", and a surface layer of more flattened cells which, in favorable sections, possessed minute microvilli over their free outer aspects. Spherical mucous cells were scattered through the epidermis. The smallest mucous cells were nearest the basement membrane; those near the surface were larger (circa 10 μ) and filled with vacuoles containing accumulations of pale basophilic mucus. Occasional cells appeared to be in the process of opening at the surface to release their content of mucus.

The deep layer of dermis consisted largely of a dense layer of collagen bundles, oriented in parallel, and containing scattered fibrocytes and blood vessels. The more superficial layer of dermis, located just beneath the basement membrane, contained a meshwork of small capillaries, melanophores, possible pigment cells of other types, fibrocytes, and a loose arrangement of collagenous fibers. Cross sections of scales were observed in sections of dermis from the trunk of the fish, but no scales were present in the fin webs from which most of the histology slides were prepared.

Skin Tumors:

Histopathologically, the fish tumors were of the following three types: angioepithelial nodule (AEN), epidermal papilloma (PAP), and angioepithelial polyp (AEP). Each tumor type revealed a different degree of interaction of the epithelial and stromal components. The AEN's were characteristically found on fish in age groups 0 and I.

Grossly, these lesions were small, 1 to 3 mm in diameter, sharply circumscribed hemispherical tumors which had a smooth surface and varied in color from white to pink to red (Figs. 1-5 and 15-16). Microscopic sections revealed that the bulk of the mass consisted of an angiomatous proliferation of vascular connective tissue surfaced by mildly hyperplastic epidermis. A continuous basement membrane separated the epidermis from the dermis (Figs. 19, 21, 28, 29). Mucous cells were usually decreased in numbers or absent. Many of the epithelial cells were similar to those of normal epidermis, while others were atypical (Figs. 28, 29). The atypical cells were larger than normal epidermal cells and ovoid in shape. Their cytoplasm was granular or finely vacuolated, nuclei stained poorly with basic dyes, and nucleoli were large, spherical, and eosinophilic. The overall appearance suggested an inflammatory response or reaction to injury rather than neoplasia. However, in the instance of the flathead sole, *Hippoglossoides elassodon*, it has been demonstrated that the AEN's transform themselves directly into typical epidermal papillomas (59).

The second type of tumor, histopathologically an epidermal papilloma, never occurred on fish in early postmetamorphosis, and in its mature form was found exclusively on fish older than 1 yr. Grossly, epidermal papillomas were generally large, 1 to 6 cm in diameter, and varied in color from grey-white to various shades of brown, to black. The centers of the lesions were raised and thrown into folds, producing a characteristic warty, cauliflower appearance (Figs. 6-14 and 17-18). At the periphery the tumors were flattened and plaque-like, gradually merging with the surrounding normal skin. Microscopically, the base

of the tumors contained much less vascular tissue than in the instance of the AEN. The epidermal component, however, was greatly thickened and composed of closely grouped papillary folds of epidermis supported by thin stalks of connective tissue stroma (Figs. 22-23, 25). Intact basement membranes separated the stromal and epidermal components (Figs. 26, 30). The epidermal component consisted mostly of ovoid (X_1) cells similar to those observed in the AEN, except that the cells attained a much larger size, especially as the free surface was approached. The cytoplasm was finely granular; vacuolated nuclei were ovoid in shape, stained poorly with basic dyes, and contained a single, large nucleolus. The supporting connective tissue contained capillaries and melanophores.

The third lesion, designated angioepithelial polyp (AEP) was previously observed on a sand sole (59), and was less frequent than the other two types. Grossly, these tumors were sessile, cauliflower-like, raised, soft, large (3 to 5 cm in diameter), and sharply demarcated from the surrounding normal skin. Microscopically, the great bulk of the tumor was composed of vascular connective tissue identical to the stromal component of the AEN (Figs. 24, 27). The epidermis was generally either apparently normal, or slightly hyperplastic. Mucous gland cells might be either present or absent from the epidermis. In some cases the epidermal cells were hypertrophic and resembled somewhat the large ovoid cells, previously described, of the epidermal papillomas.

In both the papillomas and polyps, some of the small granular cells with prominent nucleoli, located in the dermis, were morphologically very similar to some of the ovoid cells in the epidermis (Fig. 30).

Tumors were classified into one of the three types, using the gross and microscopic criteria described above. Some tumors, however, were intermediate between any two of the three types, suggesting that the small AEN tumor on the young fish of the year are possibly early forms of the larger PAP and AEP observed on older fish of age group I or greater (Chart 3). The intermediate lesions were assigned to a tumor type according to the predominant characteristics of the lesion. The AEP were infrequently observed on fish of any species.

ELECTRON MICROSCOPY

Fixation of Fish Tissue:

Several established methods used for mammalian tissues were used early in the course of this study, but the results were not satisfactory. Consequently, it was necessary to conduct preliminary fixation experiments to establish which of several possible procedures gave the most optimum results for the electron microscopy of fish skin.

Six fixation procedures were used (Table 10). Five of these procedures (Table 10, procedures 1-5) were chosen because they were known to fix adequately a great variety of tissues. The sixth procedure (Table 10, procedure 6) was chosen because it had been used successfully in an embryological study of *Fundulus heteroclitus* (99).

All six procedures were conducted concurrently using normal skin from one fish. The fixatives were also compared as to their abilities to fix tumor tissue, since it was thought possible that tumorous and normal tissue might have different fixation requirements.

Chart 3:

The histopathologic features of the three types of lesions seen on English sole and starry flounder in Bellingham Bay, Washington.

HISTOPATHOLOGY OF PLEURONECTID SKIN TUMORS

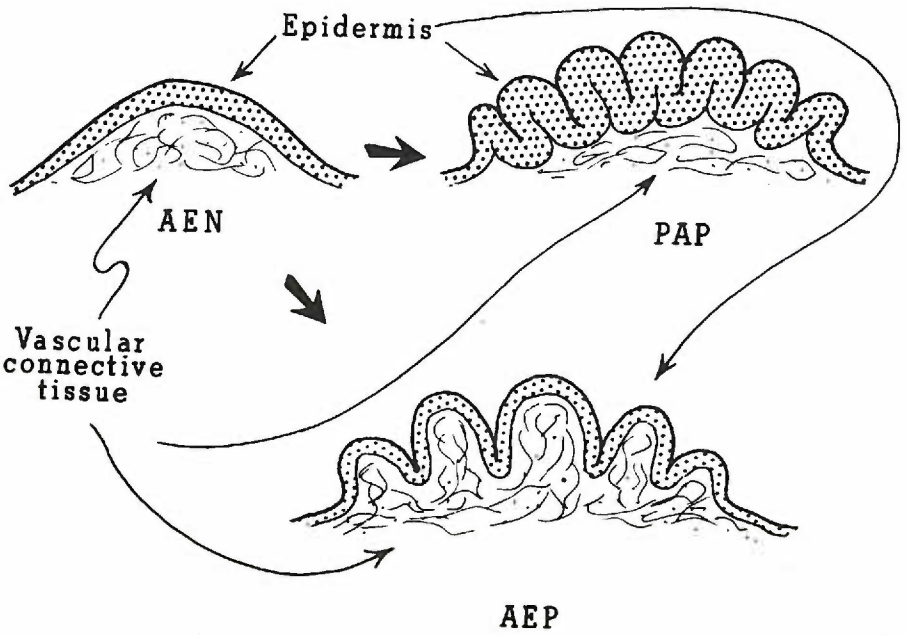


TABLE 10

COMPARATIVE FIXATION EXPERIMENT

Fixation Procedures	Maintenance of Tissue, Cells & Subcellular Organelles: Cell Membranes	Mitochondria & Subcellular Organelles	Cellular Interconnections	Fixation* Rating	Reference
1. Millonig's	+++	++	++++	++	112
2. Palay	+++++	+	++++	+++	113
3. Trump's	++++++	++	+++++	++++	114
4. Glutaraldehyde-Formalin	+	0	+	+	115
5. S-collidine	+++++	++++	++++++	+++++	100
6. Lentz	++++++	++++++	++++++	++++++	99

*Fixatives are rated + through ++++++, 6+ being the best fixative.

The fixed tissue was embedded, sectioned, and viewed by electron microscopy. The fixation procedures were rated on a basis of 1 to 6 according to their ability to preserve cell morphology. Properties such as continuity of membranes free from sharp breaks, morphology of the mitochondria, and maintenance of cellular interconnections were observed and rated (Table 10).

Of the several methods used, the procedure of Lentz (99) proved to be the best. Cellular membranes were sharp and continuous, and mitochondria and subcellular organelles (*e.g.*, ergastoplasm, Golgi apparatus, *etc.*) were well preserved.

Normal Larval Skin:

Larval starry flounders hatched following a short period of embryological development (70 hr at 11 C). The larvae were transparent and slender with a large epithelial sac (fin fold) enveloping the entire posterior portion. This fin fold, composed mostly of immature epidermis, was fixed for electron microscopy and compared to the normal and neoplastic epidermis of postmetamorphosis and mature starry flounders.

The epithelium of larval fin fold was two cells in thickness (Figs. 31-33). The cells were typically long and flattened, especially in the dorsolateral regions of the trunk. Cell margins were difficult to follow due to the numerous interdigitating surface processes. The free surface of the fin fold epithelium was sharply defined and showed surface structures similar to the microvilli found on the free surface of normal adult epidermis. The number of microvilli was less than observed at the free surface of normal adult epidermal cells. There were also fewer desmosomes than in adult stratified squamous epithelium. At

the free surface, adjacent cells were joined by a dual junctional complex (*i.e.*, a zonula occludens and one or more desmosomes) (Figs. 32-33). Interdigitation of membranes was seen which served to increase the surface-to-surface contact of adjacent cells. The actual numbers of desmosomes observed were few and seemed to be located at or near the free surface. Cytoplasmic filaments were also limited to the outer aspect of the epidermis. The nuclei in both cell layers were long, slender, and finely granular in texture; they exhibited the typical double nuclear membrane. Large cytoplasmic mitochondria containing tubular cristae were observed as were well-developed Golgi zones. Structures similar to ribosomes were observed lying free in the cytoplasm and also associated with a well-developed ergastoplasm.

Beneath the deep epidermal layer was a space 300 A in thickness (Fig. 31), limited by a continuous line which was thought to represent a primitive basement membrane. A large subdermal space filled with fluid and a finely particulate material separated the epidermis from the mesoderm. A loose arrangement of mesodermal cells was located beneath the subdermal space. Cells in both the epidermis and the mesoderm seemed to be separated by relatively large empty spaces.

Dermal components were not identified at this stage of development.

Normal Postmetamorphosis Skin:

Marked differences were observed when larval epidermis was compared to the epidermis of young starry flounders collected a few weeks after metamorphosis. The epidermal layer was now increased from two cells in thickness in the larva to three or four cells in the postmetamorphosis

form (Figs. 34-35). Microvilli were more numerous on the free surface of the epidermis and were covered by an amorphous extracellular layer averaging 130 A in thickness. Adjacent cells were joined at the free surface in the same manner as larval cells were joined; *i.e.*, by a dual junctional complex. Cytoplasmic filaments were observed in about the same numbers in the surface cells of larval epidermis, but were also present at deeper levels of the epidermis. Desmosomes were numerous and there were elaborate interdigitations of adjacent cytoplasmic membranes (Figs. 34-36).

Cells of the basal layer of the epidermis were roughly triangular in shape with the longest side adjacent to the basement membrane and parallel to the free surface. The basal cells exhibited numerous interdigitations of their cytoplasmic membranes, both with cells at the same level, and at higher levels. There were numerous desmosomes, and nuclei were finely granular and showed slight margination of chromatin. Mitochondria were numerous and varied in size and shape. A moderate quantity of both granular and agranular endoplasmic reticulum was observed.

The cells in the intermediate layers had the same type and number of cellular interconnections as observed in the basal layer. In a number of cases, however, the cytoplasmic matrix of these cells was of greater electron density. The basal cells and the cells in the layer above were equal in size and their long axes were oriented parallel to the surface. The subcellular organelles described in the basal cells were also present in the cells in the layer above, but in increased numbers.

Marked differences in cell size and shape were not noticed between the cell layers, usually four in number, in postmetamorphosis skin. At the free surface layer, a large dark cell with a tubular cytoplasmic network was observed (Figs. 34-36). This cell contained an atypical nucleus with marginated chromatin and dense, membrane-bound cytoplasmic particles.

A basement membrane separated the epidermis from the dermis. The basement membrane appeared in section as a slightly dense band measuring 200 mμ in width, with layers of compact dermal collagen below.

The dermis of the young starry flounder consisted of dense layers of collagen forming lamellae containing numerous individual collagen fibers oriented in parallel. The long axes of the fibers in each lamella were parallel to the surface, and intersected the long axis of the collagen fibers in the lamellae immediately above and below at an angle of approximately 90°. Blood vessels and parts of cells interpreted as fibrocytes were observed between the collagen lamellae.

Normal Skin; Age Group I:

The ultrastructure of skin from starry flounders of age group I was very similar to that of skin samples taken from starry flounders in the period immediately following metamorphosis. The epidermis was characterized by numerous desmosomes, cytoplasmic filaments in the upper layers, surface microvilli, various intracellular organelles similar to those previously described, and mucous cells.

The dermis was sharply separated from the epidermis by a basement membrane measuring 200 mμ in thickness (Fig. 42). The underlying dermal collagen was organized into multiple lamellae consisting of numerous

closely-packed collagen fibers oriented in parallel. As was previously described, the long axes of the collagen fibers of each lamella were parallel to the surface of the fish, and oriented at about 90° to the long axis of the collagen fibers of the lamellae immediately above and below. The individual collagen fibers showed a major longitudinal periodicity of 640 A. This corresponded approximately to the periodicity of mammalian collagen (116). Below the compact dermis was a more loosely organized layer composed of iridocytes (117), erythrophores (118), collagen bundles, and fibroblasts. The fibroblasts contained well-developed ergastoplasm and mitochondria. Beneath the fibroblasts in many cases, cells interpreted as immature melanocytes were seen.

Angioepithelial Nodule:

The epidermis of the angioepithelial nodule averaged six to eight cells in thickness (Figs. 43-44); the basal cells were normal in size (Fig. 44). Cells above the basal layers became more variable in size as the free surface was approached. The basal cells contained normal numbers of subcellular organelles (*e.g.*, mitochondria, ergastoplasm, cytoplasmic filaments, and ribosomes), compared to normal postmetamorphosis starry flounders. Interdigitations of adjacent cell membranes and desmosomes were observed in normal numbers. The long axes of the basal cells were usually perpendicular to the epidermal surface. Many cells had prominent nucleoli not observed in normal skin (Fig. 44). In the cell layers above the basal layer, mucous cells were present which were rich in ergastoplasm and contained a rather large, finely granular nucleus, many cytoplasmic vesicles, and densely staining cytoplasmic

particles (Figs. 44-45) of unknown nature. The cells of the intermediate layers of the epidermis varied in size and shape. As in normal skin, the perinuclear cytoplasm of these cells contained ergastoplasmic sacs, mitochondria, and Golgi apparatus; nuclei were pleomorphic. Surface cells were slightly flattened, but contained the usual subcellular organelles (Fig. 43). The epidermal surface showed no alteration from normal surface epidermis of the same age group.

Cells at all levels of the epidermis possessed both desmosomes and membrane interdigitations. However, interdigitations appeared to be numerically the major means of cellular interconnection (Figs. 43-44).

The basement membrane of the angioepithelial nodules was intermediate in morphology between that of the larval and adult structure (Figs. 44-46).

The dermis below the basement membrane contained isolated collagen fibers and blood vessels forming a loose vascular connective tissue, populated by several different types of cells, each type provided with many subcellular organelles (Figs. 45-46). Figure 47 illustrates cells interpreted as iridocytes and melanophores in addition to two unknown cell types (designated C_1 and X_1 in the figures and in the remainder of the text), which have large prominent nuclei. Cell C_1 contains cytoplasmic vesicles of various sizes, free and bound ribosomes, dense inclusions, mitochondria, and a Golgi complex. The nucleus of C_1 is eccentrically placed and has a prominent nucleolus. Cell X_1 also contains many vesicles, dense cytoplasmic inclusions, and free ribosomes. Its nucleus is sharply delimited and the nucleolus is large.

Figures 48 to 51 illustrate miscellaneous electron micrographs of epidermal structures at higher magnification.

Epidermal Papilloma:

The epidermis of the papilloma was greatly thickened (Figs. 52-53, 56); basal cells were broad-based, and irregular in shape (Fig. 56). The long axes of the basal layer were either parallel to, or perpendicular to, the plane of the basement membrane (Figs. 55-56). Marked membrane interdigitation was sometimes seen, but desmosomes and cytoplasmic filaments were decreased in numbers (Fig. 56). Subcellular organelles (*e.g.*, mitochondria, ergastoplasm) were normal in numbers, and were scattered throughout the cytoplasm. Ribosomes were seen unbound in the cytoplasm and also associated with the ergastoplasm.

Hypertrophic ovoid cells were distributed in considerable numbers in the suprabasal cell layers and were somewhat similar in appearance to some of the unidentified cells (X_1) formed the bulk of the tumor, were typically without intracellular attachment (desmosomes, interdigitations) to the adjacent cells, and were usually surrounded by compressed epidermal cells of relatively normal appearance. The ovoid cells had many mitochondria, lipid-like droplets, ergastoplasm, and only a few cytoplasmic filaments. The nucleus was enclosed by a double membrane with "pores", and was ovoid in shape with a prominent nucleolus. Most of the nucleoplasm appeared finely granular, evenly distributed, and of low electron density. Morphologically similar cells were sometimes seen in the underlying stroma, suggesting the possibility of invasion. In the upper levels of the epidermis, the ovoid (X_1) cells attained considerable size.

The basement membrane in cross section was a compact band measuring approximately 500 A in thickness (Fig. 56). The stroma of the epidermal papilloma consisted of loosely arranged collagenous fibers, fibrocytes, small blood vessels, and melanophores, all of which were typical by electron microscopy. In Figure 57 a cell almost identical to the ovoid (X_1) cells previously described is observed in the dermis.

Virus-like particles were observed in electron micrographs of one angioepithelial polyp (7) found on a large starry flounder (Figs. 58-59). The particles occurred in the cytoplasm of about one-half of the epithelial and stromal cells, and were organized in crystalline arrays measuring up to about 3 μ in greatest dimension. The individual particles were homogeneous, electron-dense objects of hexagonal and pentagonal outline, suggesting icosahedral symmetry. Similar closely packed particles are observed in cells infected with human enteroviruses such as polio (119) and coxsackie viruses (120).

VIROLOGICAL STUDIES

Hemagglutination Procedures:

Using the hemagglutination procedure of Clark and Casals (105), goose cells were observed to agglutinate on one occasion. In this experiment, homogenized tumor and normal tissue extracts were compared as to their ability to agglutinate goose red blood cells. The tumor tissue extract hemagglutinated cells after two hr to a titer of 320, only at 6 C and at a pH of 7.4. Titrations conducted at 20 C at several different pH's were all negative. Negative controls at both temperatures and all pH's showed the normal settling pattern. All tubes were

shaken to separate their cells and the tubes previously incubated at 20 C were incubated at 6 C, and the 6 C tubes were incubated at 20 C. All tubes remained negative, except those in which cells had previously agglutinated. These latter tubes now showed clear sparkling hemolysis, except that the lowest dilution had a settling pattern similar to the negative control. Repeated attempts to duplicate these results were made using different extracts, and using lyophilized material prepared from the one extract that had hemagglutinated the goose red blood cells. Modifications of the hemagglutination procedure, such as varying the concentration of the indicator cells, using different kinds of indicator cells (*e.g.*, chick and *H. elassodon* blood cells), varying pH and temperature, and altering the homogenization procedure, were tested without success.

Differential Centrifugation and Density Gradients:

Homogenates of tumor tissue were differentially centrifuged in an attempt to isolate virus-like particles. Normal tissue treated in the same manner served as a negative control. Initially, very simple methods of homogenization and centrifugation were used. The products of the various slow and fast spins were monitored for virus-like particles by electron microscopy with negative results. Subsequently, genetron was used to extract tissue prior to the differential centrifugation, with the hope of concentrating virus-like particles and leaving gross contaminants behind. The products of differential centrifugation were placed on sucrose or potassium citrate gradients, and further centrifuged at high speeds. Single bands were observed near

the bottom of the sucrose gradient tubes, and two bands were observed in the potassium citrate tubes. The bands were removed, stained with phosphotungstic acid, and examined by electron microscopy. Nothing suggesting virus particles was observed.

Numerous other attempts to isolate a virus-like particle were unsuccessful. In the last attempts it was assumed that a virus, if present, would have a density of about 1.30, corresponding to the heavier viruses. Gradients were spun for 45 min and 4 hr: the 45-min gradient showed five bands, and the 4-hr gradient showed as many as ten bands. Twelve-drop fractions from these gradients were stained with P.T.A. and examined in the electron microscope; nothing resembling virus was observed.

Tissue Culture:

The addition of tissue homogenates and material from density gradient bands to continuous cultures of fish cell lines resulted in no definite cytopathogenic effect (CPE) or abnormal growth of the cells. The only obvious change observed in the cell cultures was an occasional increase in cytoplasmic vacuolation which was also observed in the controls.

Transplantation Studies:

The transplantation of tumors to normal animals has not been successful, perhaps because older fish do not feed well in captivity, and the resultant poor nutritional status of the animals may favor resorption of the transplanted tumor fragments.

Transmission Studies:

Transmission studies with cell-free filtrates and "infectious" nucleic acid (106) were all unsuccessful.

DISCUSSION

Skin tumors* are common on starry flounders, *Platichthys stellatus*, and English sole, *Parophrys vetulus*, collected in Bellingham Bay, Washington. Histopathologic studies established that these tumors were essentially identical to lesions found on the flathead sole, *Hippoglossoides elassodon* (59), the rex sole, *Glyptocephalus zachirus* (59), the sand sole, *Psettichthys melanostictus* (59), and the butter sole, *Isopsetta isolepsis* (7). The tumors were of three morphologic types as follows: angioepithelial nodules, epidermal papillomas, and angioepithelial polyps. The definition of these three types of tumors in the present study was based on previous descriptions (3, 59).

The angioepithelial nodule was found predominantly on fish of the first year of life. Fish with both single and multiple tumors were observed. The tumors were 1 to 4 mm in diameter, sessile, round, and pink to red in color. Histologically, there was angiomatous proliferation of the dermal connective tissue; the overlying epidermis was slightly hyperplastic, the degree of hyperplasia increasing with the estimated age of the tumor. These tumors appeared similar to angioepithelial nodules described on *Hippoglossoides elassodon*.

---

*The word "tumor" as used in this thesis is synonymous with neoplasm. The well known definition of tumor (neoplasm) given by Willis (121) is as follows: "A tumor is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues, and persists in the same excessive manner after cessation of the stimuli which evoked the change." Hyperplasia, on the other hand, is a more coordinated cell proliferation which ceases or regresses when the responsible stimulus is removed.

Tumors were first observed on English sole of approximately five to six months of age, collected from the *M. V. Hydah*. Very few starry flounders collected from the *Hydah* were in the 0 age group and fewer still were afflicted with the angiomatous lesion. Beach seine collection data were in sharp contrast to collection data obtained from the fishing vessel. As mentioned previously, starry flounders of age group 0 collected from the *Hydah* were few in number. However, numerous very young (postmetamorphosis) starry flounders, measuring 1 to 4 cm in total length, were collected by beach seine on the southwest side of the Nooksack River Estuary.

The incidence of angioepithelial nodules in these juvenile starry flounders was very high: 54 per cent had one to several angioepithelial nodules. This incidence exceeded that at any other collecting site or in any other age group. The beach seine collections contained only two species of fishes; namely, starry flounders and staghorn sculpins, *Leptocottus armatus*. Both species are known to be euryhaline. The Nooksack River empties into Bellingham Bay a short distance from the locations where fish were collected by beach seine and is responsible for the low salinity of the inshore water at low tide. English sole were not observed in the Nooksack Estuary, perhaps because that species cannot tolerate such low salinity, which at times is essentially that of fresh water.

Gravid female and ripe male starry flounders were observed in the months of March and April. Tumorous postmetamorphosis starry flounders of age group 0 were collected in the months of July through September. This indicates that these fish were approximately three to four months

old when captured. Even at this age the nodules were well-developed and occurred as single or multiple tumors.

Fertilized eggs develop into embryos which hatch in approximately 5 days in the laboratory at 11 C*. At this time they are approximately 2.0 mm in total length. The larva of the starry flounder probably remains pelagic until it attains a standard length of approximately 10.0 mm. At this time metamorphosis occurs, and the young fish assume the form and habit of miniature bottom dwelling adults (122). The smallest tumorous specimen collected with a beach seine was 27.0 mm in standard length.

Chi-square tests indicate that the predominance of tumors on the pigmented (eye) sides of age group 0 fish (both dextral and sinistral starry flounder collected by beach seine, and English sole, collected from the fishing vessel) are statistically significant. Pelagic pleuronectid larvae are bilaterally symmetrical prior to metamorphosis, and the right and left sides are identical except that they are mirror images of each other. During metamorphosis the larvae become asymmetrical with their pigmented (eye) side uppermost with respect to the substratum, leaving the nonpigmented (blind) side lowermost with respect to the substratum.

As has been pointed out previously (3), the difference in attack rate on the two sides suggests that the tumorigenic event, or events, occur sometime after metamorphosis, since prior to that time the two sides are essentially identical. Since starry flounder metamorphosis

---

*McArn, G. E., unpublished observations.

occurs when the larva reaches about 10 mm in standard length, it would be expected, therefore, that the tumorigenic event(s) might occur sometime after the larva reaches 10.0 mm and before the fish reaches 30.0 mm in standard length, because tumors are known to occur at 30 mm. This would narrow the interval of tumorigenesis to a 2- to 3-month period during the summer months. The actual age at which metamorphosis occurs is unknown.

The same line of reasoning applies to English sole of age group 0. Though the fish were older, the angioepithelial nodules were most numerous during the months of September and October and decreased in number in the following months. As the number of angioepithelial nodules decreased, the number of transitional tumors and epidermal papillomas increased, suggesting that the angioepithelial nodules evolve into epidermal papillomas. The tumor incidence in English sole of age group 0 was lower than that in starry flounders of the same age group.

Both English sole and starry flounders of age group I or older are afflicted with typical papillomas. These tumors are large cauliflower-like lesions (1-4 cm in diameter) which are grey to brown to black in color. They are characterized by a marked papillomatous proliferation of the epidermis, supported by a fine, poorly vascularized connective tissue stroma. At least some tumors progressively enlarge in the laboratory, but metastases have never been observed. Similar tumors have been previously described on *Psettichthys melanostictus* (3, 5), *Parophrys vetulus* (1, 2, 7), *Limanda limanda* (20), *Glyptocephalus zachirus* (3), and the starry flounder (7).

English sole with typical epidermal papillomas are most numerous in the month of February. The number of tumor-bearing fish decreases both before and after this month. Only on three occasions were tumorous fish of age group I not taken in the collections. Only epidermal papillomas were observed in English sole of age group I or greater; new lesions were never observed to appear in the laboratory.

Starry flounders in age groups I or older generally have solitary epidermal papillomas. As mentioned in the results, chi-square tests indicate that tumor distribution according to side (pigmented versus nonpigmented) is not significant; however, earlier work strongly suggested that there was a preferential tendency for tumors to occur on the pigmented side (7). Papillomas were found on starry flounders in the months of October through May. After this time, tumorous starry flounder were very infrequent. Age groups II and III fish were occasionally collected and their tumors were solitary and often large.

The angioepithelial polyps are not frequently found on either the starry flounder or the English sole. In contrast to most epidermal papillomas, the large, raised, soft angioepithelial polyps are sharply demarcated from the surrounding skin at the periphery of the tumor. Microscopically, the bulk of the tumor consists of vascular connective tissue surfaced by normal or slightly hyperplastic epidermis, with or without mucous cells. Frequently, some portions of the epidermis possess cells which are enlarged and resemble those cells typically found in the thickened epidermal folds of the papilloma. The angioepithelial polyps occur in fish of age group I or older.

In both the papillomas and the polyps, there are ovoid cells located in the dermis which are granular and contain large nucleoli, and which are morphologically similar to some of the cells found in the epidermis and assumed to be epithelial cells. A satisfactory explanation for this observation was not evident.

There is evidence that the angioepithelial nodules evolve into epidermal papillomas or angioepithelial polyps. Firstly, the nodules are most frequent on the smallest fish and youngest fish, and the papillomas and angioepithelial polyps are observed on older fish. Secondly, numerous transitions between the tumor types are observed, both grossly and microscopically, during the winter months, suggesting a histogenetic relationship. Thirdly, angioepithelial nodules of at least one species of pleuronectid, the flathead sole, have been observed to evolve directly into typical epidermal papillomas under laboratory conditions (3). The proposed histogenetic relationships are schematically represented in Chart 3.

It is not known whether tumorous fish die of the disease, or recover, or whether either event may occur. It seems reasonable to assume that fish with massive, progressive lesions such as illustrated in Figure 9 are less maneuverable, possibly less successful in obtaining food, and more susceptible to predation, than normal fish. Field observations indicate that tumorous fish may be less robust and succumb to the trauma of the collection net much more readily than healthy normal fish. Future morphometric and survival studies will be necessary to determine the fate of tumor-bearing fish.

The natural history data can be summarized as follows: spawning in both species probably occurs in late winter and early spring, the fertilized eggs develop in the planktonic habitat, and the eggs hatch in perhaps 5 to 10 days, depending on the temperature. The larvae may be planktonic for 3 to 5 months (122), after which they metamorphose and appear on mud-sand flats, where they may be collected by beach seine in July and August. At this time, early tumors (AEN) are already present, predominantly on the pigmented side, suggesting that the tumorigenic agent(s) act sometime after metamorphosis, and have a predilection to affect the pigmented side more often than the non-pigmented side, for reasons which are unknown.

The "nursery" areas for the very young fish following metamorphosis are shallow, inshore, protected mud-sand flats characterized by higher water temperature and often by relatively low salinity. The fish collected during late fall and early winter show the temporal sequential appearance of angioepithelial nodules, transitions between nodules and epidermal papillomas, and finally (in early winter), mature epidermal papillomas. Angioepithelial nodules were never found on fish older than one year, nor did new lesions ever appear on fish of any age group in the laboratory. The sum total of the natural history data thus suggests that there is a yearly cycle of tumorigenesis in the English sole and starry flounder. This cycle appears to correspond closely to that observed over a period of at least 6 years in flathead sole collected in East Sound of Orcas Island (3, 8, 59).

Whether or not the epidermal papillomas are true neoplasms is an unanswerable question at present, since the papillomas have not been sufficiently analyzed under controlled laboratory conditions. It seems likely that the lesions are neoplastic because: 1) the complexity of the papillomatous interaction of stroma and epithelium exceeds that seen in the usual hyperplasias, and 2) at least some tumors progressively enlarge under laboratory conditions.

Several authors have described somewhat similar papillomatous proliferations of epidermis occurring in a variety of species of fishes, including some pleuronectids (33, 38, 42, 43, 47). These lesions have often been designated "hyperplastic epidermal disease" or "fish pox" (47). Schlumberger and Lucké (41) state that there is no sharp histological distinction between hyperplastic epidermal disease and true neoplasia. Generally speaking, the former is capable of regression, while the latter is not. Nigrelli (43) has never observed hyperplastic epidermal disease in the absence of some sort of parasitic skin infestation and suggests a possible etiological role for parasites. Although lymphocystis disease, caused by a virus, produces papillomatous tumor-like dermal masses in various species of fishes, including flounders, the disease described in this thesis bears no histological resemblance to lymphocystis disease (123).

Spontaneous tumors are known to occur in all five classes of vertebrates, in cephalochordates, mollusca, annelids, and arthropods. Experimental induction of tumors has been achieved in mammals, birds, amphibians, insects, and crustaceans (124). Several instances of genetically determined tumors in fish, insects, higher plants, and inbred lines of mammals are also known.

Spontaneous neoplasms have been reported in at least 121 species of fishes (41) and a number of species of amphibians (125). In these classes, tumors of skin and subcutaneous tissue are most common. The few reported spontaneous tumors in reptiles are mostly melanomas (41).

Willis (121) indicates that the incidence of spontaneous tumors generally increases as the physiological age of a population of animals increases. This generalization apparently does not apply to the disease described herein, since the data indicate that tumor incidence is highest in the youngest fish, and decreases progressively as age increases. The operation of an infectious process in the genesis of skin tumors in the starry flounder and English sole is suggested by the high incidence in crowded populations of young individuals, and by seasonal annual cycles of the disease.

The etiology of the disease is unknown. The induction of the tumors by tumorigenic virus(es) is a possibility which needs to be further explored. In other species of fish, various protozoa, helminths, and other parasites are associated with hyperplastic epidermal disease and have been proposed as etiological agents (33, 38, 42, 43, 47). In this regard, Nigrelli (43) states that, "Intercellular and intracellular cnidosporidians elaborate proteolytic enzymes and other chemical substances which may be responsible for considerable cellular degeneration, cell hypertrophy and other tissue responses noted in the infection." Some of these responses are clearly proliferative and may resemble or border on neoplasia.

Moreover, Smith (33) observed that winter flounder, *Pseudopleuronectes americanus*, with hyperplastic epidermal disease were also

infected with metacercarial larvae of a digenetic trematode, *Cryptocotyle lingua*.

The association of the ciliates *Trichodina* and *Ichthyophtherius* with hyperplastic epidermal disease in freshwater bluegill sunfish has been reported (43). The latter ciliate causes thickening of both components of the skin, and normally feeds on the host tissue cells. The bluegill sunfish is particularly susceptible to this ectoparasite during the months when water temperatures show maximum variation. The parasite enters the skin and may be overgrown by the epithelium. Whether this encystment of the parasite is a cause of hyperplasia is at present unknown. It is at least generally true that encysted parasites frequently cause reactive hyperplasia of host connective tissue in a wide variety of animals, both terrestrial and aquatic.

Schaperclaus (126) and Wolfe (127) both suggest that nutrition may be responsible for, or contribute to the development of, hyperplastic epidermal disease. The latter investigator found that trout gill disease, a hyperplasia produced by irritants, was related to dietary deficiency in pantothenic acid.

Another possibility is that the associated parasites function as vectors for viral transmission with the virus being the agent responsible for the subsequent development of hyperplasia or neoplasia. Diseases such as fish pox, lymphocystis disease, and hyperplastic epidermal disease have been considered to be caused by viruses. Loewenthal (128) in 1907 found inclusion bodies in the epithelial cells from hyperplastic epidermal disease of European cyprinids. It is known that parasites do transmit viral diseases in higher animals, *e.g.*,

salmon poisoning and swine influenza. In both cases the larval forms of parasites transmit the viral infection.

It follows from the preceding discussion that multiple environmental factors could contribute to the high incidence of epizootic skin neoplasia observed in pleuronectids in Bellingham Bay. These multiple environmental agents might well include any one or several of the following: tumorigenic viruses, ectoparasitic vectors, carcinogenic agents in the substratum or water, and the presence of mechanically or chemically irritative incident particulates. The possibility of susceptible genomes in certain species, subspecies, or populations of fishes must also be considered.

That there is a seasonal variation in the physical (*e.g.*, temperature), chemical (*e.g.*, salinity, pollutants), and biological properties (*e.g.*, biological pollutants) of the environment seems incontrovertible. It appears that the combination of environmental circumstances on certain sand-mud flats in Bellingham Bay may critically favor tumorigenesis in the species concerned during mid- and late summer. Ectoparasitic infestation of young fish on the mud flats of the Nooksack estuary in the summer months is documented herein, but no etiological or pathogenetic relationship is demonstrated.

Inasmuch as virus-like particles were observed on some of the tumors by electron microscopy, efforts were made to isolate biologically active viral agents from the neoplasms. These methods included several variations of extraction procedures, and of differential centrifugation with and without density gradients. In addition, extracts were examined by electron microscopy. In no case was there any evidence

of a viral agent in the extracts.

The most convincing evidence for the presence of a viral agent was observed by electron microscopy of thin sections of one angioepithelial polyp found on a starry flounder. The particles, measuring 300 A, were present in huge numbers, oriented in crystalline array in both epithelial and stromal cells, and corresponded in morphology to some enteroviruses. However, they were unlike the virus-like particles observed in the papilloma of the flathead sole (4, 59, 60). No evidence was obtained to indicate that these virus-like particles are of etiological significance.

Normal skin and tumors from starry flounders were compared by electron microscopy, in search of differences which might suggest etiologic or pathogenetic mechanisms. Normal skin was studied from larval fish, from fish of the year in the immediate period after metamorphosis, and from fish in the second year of life.

The two-layered epithelium of the fin fold of the larval flounder was characterized by well formed mitochondria, well-developed Golgi apparatus, ergastoplasm, and numerous ribosomes. The presence of these subcellular components suggests active protein synthesis. Although no mucous cells were present in the larval epidermis, differentiation of cytoplasmic filaments similar to those of older fish was observed in the outer cell layer. The basement membrane was apparently represented by a linear band measuring 100 A in thickness and located just beneath the two-layered epithelium. The characteristic dermal components such as collagen and pigment cells were not observed in larval epidermis.

An important feature of the epidermis may be the relatively loose manner in which basal cells and mesodermal cells were adjoined. The thinness of the primitive basement membrane and the loose junction of

adjacent cells may serve to facilitate diffusion; nutrients from the yolk-sac, diluted in the fluid of the subdermal space, may thus diffuse more readily across the primitive basement membrane and through the intercellular spaces to the epidermal cells.

Juvenile starry flounders collected shortly after metamorphosis exhibited a very highly differentiated cutaneous structure. The layers in the epidermis had increased in number from 2 to 4 over that of the larval condition. Structures at all levels of the epidermis were well differentiated with typical mucous cells, desmosomes, interdigitations of the cell membranes, and cytoplasmic filaments. Basement membranes had taken on the form observed in older fish. Collagen and blood vessels were recognized in the dermis.

The epidermis of starry flounders of age group I was 6 or more cells thick, but was otherwise identical to that of the juvenile starry flounder; the dermis contained melanophores, iridocytes, and collagen bundles.

The following can be concluded from this study: 1) the epidermal cells and the basement membrane may develop before the dermis, suggesting that the basement membrane is of epidermal origin; 2) the mucous cells and epidermal cells with cytoplasmic filaments and desmosomes differentiate largely after metamorphosis; and 3) dermal components such as collagen, pigment cells, and blood vessels were seen after metamorphosis, but not in the early larval stages.

The epidermal papillomas contained an abundance of ovoid (X_1) cells which did not resemble the cells of normal epidermis. These cells were often several times larger than normal epidermal cells, and showed swollen

vesicular mitochondria with decreased numbers of cristae, very large nucleoli, dense cytoplasmic granules, and an absence of desmosomal attachments to surrounding compressed epidermal cells. Morphologically similar cells were sometimes observed in the stroma of the epidermal papillomas, and they were possibly also present in the epithelial and stromal components of both the angioepithelial nodule and angioepithelial polyp.

The nature of the hypertrophic ovoid (X_1) cell is unknown. It seems most likely that they are transformed fish cells of either dermal or epidermal origin. It is noteworthy that similar cells were not found in any normal fish tissue examined. Another possibility is that the hypertrophic ovoid cell is a parasitic cell, possibly protozoal. If the ovoid cell is parasitic, then it represents a form heretofore undescribed.

In conclusion, the present study has elucidated the broad outlines of the natural history of epidermal papillomas and related skin lesions in the starry flounder and English sole, and has described histopathologic and ultrastructural characteristics in both normal and tumorous skin in considerable detail. Preliminary attempts were made to transmit the disease, transplant the tumors, and isolate an etiologic agent. Although these latter studies were without positive results, they are far from conclusive. Further studies of the biology of this disease should utilize a combination of experimental, virological, and ecological techniques.

SUMMARY

A study of the natural history of skin tumors occurring in two species of Pleuronectidae was conducted in the waters of Bellingham Bay, Washington. Twelve collections at monthly intervals indicated that 4.8 per cent of 1,977 English sole (*Parophrys vetulus*) and 2.4 per cent of 2,946 starry flounders (*Platichthys stellatus*) were tumorous. Three types of tumors were observed, which were classified as angioepithelial nodule, epidermal papilloma, or angioepithelial polyp, according to nomenclature previously established by other workers. The angioepithelial nodule was found on fish in their first year of life and not convincingly on fish older than one year. Epidermal papillomas usually occurred on fish which were one year or older, as did the angioepithelial polyp. The incidence of tumor-bearing fish and the average number of tumors on a single specimen was greatest during the first year of life and decreased progressively in older age groups. Tumors were more likely to occur on the pigmented side than on the nonpigmented side of the fish.

Ultrastructural studies of normal larval skin revealed epidermal desmosomes, a few cytoplasmic filaments on the surface cells, surface microvilli, and a thin primitive basement membrane, but no recognizable differentiated dermal components. Skin from juvenile starry flounders collected in the period shortly after metamorphosis showed well-differentiated epidermal structures of all types, as well as differentiation of dermal collagen, pigment cells, and blood vessels. Skin of starry flounders in the first year of life was similar to skin of the juvenile fish.

Convincing virus-like particles were observed in only one angio-epithelial polyp from a starry flounder. Attempts to isolate an etiological agent from a number of different tumors were unsuccessful.

REFERENCES

1. Pacis, M. R. An epithelial tumor of *Parophrys vetulus*. Unpublished master's thesis. Univ. Washington, 1932.
2. Good, H. V. A study of an epithelial tumor of *Parophrys vetulus*. Unpublished master's thesis. Univ. Washington, 1940.
3. Wellings, S. R., Chuinard, R. G., Gourley, R. T., and Cooper, R. A. Epidermal papillomas in the flathead sole, *Hippoglossoides elassodon*, with notes on the occurrence of similar neoplasms in other pleuronectids. J. Nat. Cancer Inst., 1964. 33, 991-1004.
4. Wellings, S. R., and Chuinard, R. G. Epidermal papillomas with virus-like particles in flathead sole, *Hippoglossoides elassodon*. Science, 1964. 146, 932-933.
5. Nigrelli, R. F., Ketchen, K. S., and Ruggieri, G. D. Studies on virus diseases of fishes. Epizootiology of epithelial tumors in the skin of flatfishes of the Pacific Coast, with special reference to the sand sole (*Psettichthys melanostictus*) from northern Hecate Strait, British Columbia, Canada. Zoologica, 1965. 50, 115-122.
6. Chuinard, R. G., Berkson, H., and Wellings, S. R. Surface tumors of starry flounder and English sole from Puget Sound, Washington. Fed. Proc., 1966, 25, 661. (Abstract)
7. McArn, G. E., Chuinard, R. G., Miller, B. S., Brooks, R. E., and Wellings, S. R. The pathology of skin tumors found on English sole and starry flounder from Puget Sound, Washington. J. Nat. Cancer Inst. In press.

8. Chuinard, R. G. The natural history and pathology of epidermal papillomas of flathead sole, *Hippoglossoides elassodon*, from East Sound of Orcas Island, Washington. Unpublished master's thesis. Univ. Oregon Medical School, 1966.

9. Hegner, R. W., and Stiles, K. A. College Zoology (6th Ed.). New York: MacMillan, 1956. pp 388-403.

10. Stolk, A. Chromaffinoma in amphioxus. Proc. Kon. Ned. Akad. Wet., 1961. 64C, 478-488.

11. Lucké, B., and Schlumberger, H. G. Neoplasia in cold-blooded vertebrates. Physiol. Reviews, 1949. 29, 91-126.

12. McFarland, D. J. Epithelioma of the mouth and skin of a catfish. Proc. Path. Soc. Phila., 1901. 4, 79-81. (Cited by Schlumberger and Lucké, 1948.)

13. Dauwe, F., and Pennemann, G. Contributions à l'étude du cancer chez les poissons. Ann. Soc. méd. Gand, 1904. 84, 81-90. (Cited by Schlumberger and Lucké, 1948.)

14. Bashford, E. F., Murray, J. A., and Cramer, W. Transplantation of malignant new growths. Sci. Repts. Invest. Imp. Cancer Res. Fund, 1905. 2, 13-17.

15. Keysselitz, G. Über ein Epithelioma der Barben. Arch. f. Protistenkunde, 1908. 11, 326-333. (Cited by Schlumberger and Lucké, 1948.)

16. Murray, J. A. The zoological distribution of cancer. Sci. Repts. Imp. Cancer Res. Fund, 1908. 3, 41-60.

17. Schroeders, V. D. Tumors of fishes. Dissertation (Russian). (Translation in Army Medical Library, Washington, D. C.) St. Petersburg, 1908. (Cited by Schlumberger and Lucké, 1948.)

18. Fiebiger, J. Über Hautgeschwülste bei Fischen nebst Bermerkungen über die Pockenkrankheit der Karpfen. Ztschr. f. Krebsforsch., 1909. 7, 165-179. (Cited by Schlumberger and Lucké, 1948.)

19. Clunet, J. Recherches expérimentales sur les tumeurs malignes. Paris, 1910. (Cited by Schlumberger and Lucké, 1948.)

20. Johnstone, J. Internal parasites and diseased conditions of fishes. Proc. and Trans. Liverpool Biol. Soc., 1912. 26, 103-104.

21. Beatti, M. Geschwülste bei Tieren. Ztschr. f. Krebsforsch., 1916. 15, 452-491. (Cited by Schlumberger and Lucké, 1948.)

22. Breslauer, T. Zur Kenntnis der Epidermoidalgeschwülste von Kaltblütern. Arch. f. Mikroskp. Anat., 1916. 87, 200-264. (Cited by Schlumberger and Lucké, 1948.)

23. Johnstone, J. Diseased conditions in fishes. Report, Lancashire Sea-Fisheries Lab., 1924. No. XXXII, 99-129.

24. Anitschkov, N., and Pavlovsky, E. N. Über die Hautpapillome bei Gobius u. ihre Beziehung zur Normalen Struktur der Fischhaut. Ztschr. f. Krebsforsch., 1923. 20, 128-147.

25. Johnstone, J. Diseased conditions in fishes. Report, Lancashire Sea-Fisheries Lab., 1925. No. XXXIII, 105-136.

26. Sagawa, E. Zur Kenntnis der Fischengeschwülste. Gann, 1925. 19, 14-15. (Cited by Schlumberger and Lucké, 1948.)

27. Takahashi, K. Studie über Fischtumoren. Ztschr. f. Krebsforsch., 1929. 29, 1-73.

28. Williams, G. Tumorous growths in fish. Proc. and Trans. Liverpool Biol. Soc., 1929. 43, 120-148.

29. Thomas, L. Contribution à l'étude des lesions précancereuses chez les poissons. Les papillomes cutanées de la sole. Bull. Assoc. franc l'étude Cancer, 1930. 19, 91-97.

30. Thomas, L., and Oxner, M. Papillomes de la lèvre inférieure chez *Anguilla vulgaris*. Bull. Assoc. franc l'étude Cancer, 1930. 19, 708-714.

31. Puente-Duany, N. Tumoracion periocular en un pescade de rio. Bol di liga contra el cancer, 1930. 5, 240. (Abstract in Am. J. Cancer, 1931; 15, 981.)

32. Thomas, L. Papillome tégumentaire chez un truite. Bull. Assoc. franc p. l'étude Cancer, 1932. 21, 547-550.

33. Smith, G. M. A hyperplastic epidermal disease in the winter flounder infected with *Cryptocotyle lingua* (Creplin). Am. J. Cancer, 1935. 25, 108-112.

34. Coates, C. W., Cox, R. T., and Smith, G. M. Papilloma of the skin in an electric eel, *Electrophorus electricus*. Zoologica, 1938. 23, 247-251.

35. Lucké, B. Studies on tumors in cold-blooded vertebrates. Ann. Rept. Tortugas Lab., Carnegie Inst. Wash. D. C., 1938. 1937-1938, 92-94.

36. Nigrelli, R. F., and Smith, G. M. A papillary cystic disease affecting the barbels of *Ameiurus nebulosus* (Le Sueur), caused by the myxosporidian *Henneguya ameiurensis* sp. nov. Zoologica, 1940. 25, 89-96.

37. Lucké, B., and Schlumberger, H. G. Transplantable epitheliomas of the lip and mouth of catfish. 1. Pathology. Transplantation to anterior chamber of eye and into cornea. J. Exper. Med., 1941. 74, 397-408.

38. Nigrelli, R. F. Studies of the marine resources of southern New England. V. Parasites and diseases of the ocean pout, *Macrozoarcus americanus*. Bull. Bingham Oceanographic Collection, 1946. 9, 187-221.

39. Smith, G. M. Fibro-epithelial growths of the skin in large marine turtles, *Chelonia mydas*. Zoologica, 1938. 23, 93-98.

40. Christiansen, M., and Jensen, A. J. C. On a recent and frequently occurring tumor disease in eel. Reports Danish Biological Sta., 1947. No. 50, 29-44.

41. Schlumberger, H. G., and Lucké, B. Tumors of fishes, amphibians, and reptiles. Cancer Res., 1948. 8, 657-712.

42. Nigrelli, R. F. Prickle cell hyperplasia in a snout of the redhorse sucker (*Moxostoma aureolum*) associated with an infection by the myxosporidian *Myxobolus moxostomi* sp. nov. Zoologica 1948. 33, 43-46.

43. Nigrelli, R. F. Hyperplastic epidermal disease in the blue gill sunfish, *Lepomis macrochirus* Rafinesque. Zoologica, 1948. 33, 133-137.

44. Aronowitz, O., Nigrelli, R. F., and Gordon, M. A spontaneous epithelioma in the platyfish, *Xiphophorus (Platypoecilus) variatus*. Zoologica, 1951. 36, 239-242.

45. Tavolga, W. N. Epidermal fin tumors in the gobiid fish, *Bathygobius soporator*. Zoologica, 1951. 36, 273-278.

46. Ghadially, F. N., and Whiteley, H. J. Hormonally induced epithelial hyperplasia in the goldfish (*Carassius auratus*). Brit. J. Cancer, 1952. 6, 246-248.

47. Nigrelli, R. F. Virus and tumors in fishes. Ann. N. Y. Acad. Sci., 1952. 54, 1076-1092.

48. Sarkar, H. L., and Dutta-Chaudhuri, R. On the occurrence of an epidermal papilloma in Koi fish, *Anabas testudenius* (Bloch). J. Indian Med. Ass., 1953. 22, 152-154.

49. Stolk, A. Tumours of fishes. III. Carcinoma of the epidermis in the black variety of the viviparous cyprinodont *Xiphophorus hellerii* Heckel. Proc. Kon. Ned. Akad. Wet., 1953. 56C, 143-148.

50. Ermin, R. On an ocular tumor with exophthalmia in an interspecific hybrid of anatolichthys. Rev. Fac. Sci. Univ. Istanbul, 1954. Series B. Sci. Nat., 19, 203-211.

51. Stolk, A. Tumours of fishes. IXA. Epithelioma of the oral mucosa in the scyllid, *Scylliorhinus catulus*. Proc. Kon. Ned. Akad. Wet., 1956. 59C, 196-210.

52. Stolk, A. Tumours of fishes. XI. Carcinoma of the skin in the anabantid *Colis labiosa* (Day). Proc. Kon. Ned. Akad. Wet., 1956. 59C, 624-633.

53. Russell, F. E., and Kotin, R. A. Squamous papilloma in the white croaker. J. Nat. Cancer Inst., 1957. 18, 857-860.

54. Stolk, A. Tumours of fishes. XXII. Epidermoid carcinoma of the upper lip in the characid *Ephippicharax orbicularis* (Valenciennes). Proc. Kon. Ned. Akad. Wet., 1958. 61C, 201-206.

55. Imai, T., and Fujiwara, N. An electron microscopic study of a papilloma-like hyperplastic growth in a goby, *Acanthogobius flavimanus*. Kyushu J. Med. Sci., 1959. 10, 135-147.

56. Stolk, A. Tumours of fishes. XXXI. Epidermoid carcinoma in a strain of the cichlid, *Etroplus maculatus* (Bloch). Proc. Kon. Ned. Akad. Wet., 1960. 63C, 200-219.

57. Walker, R. Fine structure of a virus tumor in fish. American Zoologist, 1961. 1, 395-396.

58. Moore, D. H., Lasfargues, E. Y., Murray, Margaret R., Cushman, D. H., and Pollard, E. C. Correlation of physical and biological properties of mouse mammary tumor agent. J. Biophys. Biochem. Cytol., 1959. 5, 85-92.

59. Wellings, S. R., Chuinard, R. G., and Bens, M. A comparative study of skin neoplasms in four species of pleuronectid fishes. Ann. N. Y. Acad. Sci., 1965. 126, 497-501.

60. Wellings, S. R., Chuinard, R. G., and Cooper, R. A. Ultrastructural studies of normal skin and epidermal papillomas of the flathead sole, *Hippoglossoides elassodon*. Zschr. Zellforsch., 1967. 78, 370-387.

61. Chuinard, R. G., Berkson, H., and Wellings, S. R. Surface tumors of starry flounder and English sole from Puget Sound, Washington. Fed. Proc., 1966. 25, 661. (Abstract)

62. Wellings, S. R., Cooper, R. A., and Chuinard, R. G. Skin tumors of pleuronectid fishes in Puget Sound, Washington. Bulletin of the Wildlife Disease Association, 1966. 2, 68.

63. Kimura, I., Sugiyama, T., and Ito, Y. Papillomatous growth in sole from Wakasa Bay area. Proc. Soc. Exp. Biol. Med., 1967. 125, 175-177.

64. Jézéquel, A.-M., and Steiner, J. W. Some ultrastructural and histochemical aspects of Coxsackie virus-cell interactions. Lab. Invest., 1966. 15, 1055-1083.

65. Murray, J. A. The zoological distribution of cancer. Sci. Repts. Imp. Cancer Res. Fund, 1908. 3, 41-60.

66. Champy, C., and Champy, Mlle. Sur am epithelioma transmissible chez le triton. Bull. Assoc. franc p. l'étude Cancer, 1935. 24, 206-220.

67. Sheremetieva-Brunst, E. A., and Brunst, V. V. Origin and transplantation of a melanotic tumor in the axolotl. IN The Biology of Melanomas, Spec. Pub. N. Y. Acad. Sci., 1948. 4, 269-287.

68. Rickenbach, J. Über ein spontan enslandenes Carcinom bei *Triton alpestris*. Rev. Suisse pathol. gén. bacteriol., 1950. 13, 497-503.

69. Sheremetieva-Brunst, E. A. A further investigation of melanotic tumors in the axolotl, *Siredon mexicanum*. Cancer Res., 1952. 12, 296.

70. Stolk, A. A transplantable fibroma of the skin in the newt, *Triturus taeniatus*. Experientia, 1958. 14, 243.

71. Eberth, C. J. Multiple Adenome der Froschhaut. Virch. Arch. f. path. Anat., 1868. 44, 12-22.

72. Murray, J. A. The zoological distribution of cancer. Sci. Rept. Imp. Cancer Res. Fund, 1908. 3, 41-60. (Cited by Schlumberger and Lucké, 1948.)

73. Pavlovsky, E. N. Zur Kasuistik der Tumoren beim Frosch. Centralbl. f. allg. Path. u. path. Anat., 1912. 23, 94.

74. Pentimalli, F. Über die Geschwülste bei Amphibien. Ztschr. f. Krebsforsch., 1914. 14, 623-632.

75. Secher, K. Kasuistische Beiträge zur Kenntnis der Geschwülste bei Tieren. Ztschr. f. Krebsforsch., 1917/1919. 16, 297-313.

76. Masson, P., and Schwartz, E. Un cas d'épithélioma cutané chez le grenouille verte. Bull. Assoc. franc p. l'étude Cancer, 1923. 12, 719-725.

77. Duany, J. Un epithelioma glandular en una *Rana* bull frog. Arch. Soc. Estud. Clin. Habana, 1929. 29, 186-195.

78. Perlot, J. M., and Welsch, M. Étude anatomique et expérimentale de quelques tumeurs chez la grenouille rousse (*Rana fusca*). Arch. Internat. d. Méd. Expér., 1934. 9, 341-356.

79. Llambés, J. J., and Garcia, J. M. Aparicion espontanea de tumores multiples en la *Rana catesbiana*. Rev. med. Cubana, 1949. 60, 26-32.

80. Stolk, A. Skin adenoma in *Rana arvalis* Nilson. Proc. Kon. Ned. Akad. Wet., 1957. 60C, 167-183.

81. Stolk, A. Guanophoroma in the frog *Hyla arborea meridionalis*. Proc. Kon. Ned. Akad. Wet., 1959. 62C, 390-395.

82. Stolk, A. Xanthophoroma in the frog *Hyla arborea*. Proc. Kon. Ned. Akad. Wet., 1959. 62C, 568-575.

83. Stolk, A. Erythrophoroma in *Dendrobates typographicus*. Proc. Kon. Ned. Akad. Wet., 1959. 62C, 381-389.

84. Stolk, A. Development of the cutaneous fibroma of the adepidermal reticular network in the skin of the toad *Bufo bufo japonicus*. Acta Neerl. Morphol., 1961. 4, 237-253.

85. Elkan, E. A. Three different types of tumors in salientia. Cancer Res., 1963. 23, 1641-1645.

86. Lucké, B. Studies on tumors in cold-blooded vertebrates. Ann. Rept., Tortugas Lab., Carnegie Inst., Wash., 1938. 1937/38, 92-94.

87. Smith, G. M. The occurrence of trematode ova, *Hapalotrema constrictum*, in fibroepithelial tumors of the marine turtle, *Chelonia mydas*. Zoologica, 1939. 24, 379-389.

88. Nigrelli, R. F. The occurrence of leeches, *Ozobranchus branchiatus*, on fibro-epithelial tumors of marine turtles, *Chelonia mydas*. Zoologica, 1943. 28, 107-108.

89. von Hansemann, D. Discussion. Verhandl. d. Berlin med. Gesellschaft, 1906. 36, 231-232. (Cited by Schlumberger and Lucké, 1948.)

90. Koch, M. Demonstration einiger Geschwülste bei Tieren. Verhandl. d. Deut. Path. Gesell., 1904. 7, 136-147.

91. Heller, J. Discussion. Verhandl. d. Berlin med. Gesellschaft, 1906. 36, 230-231. (Cited by Schlumberger and Lucké, 1948.)

92. Plehn, M. Über Geschwülste bei Kaltblütern. Wien. klin. Schnschr., 1912. 25, 691-697. (Cited by Schlumberger and Lucké, 1948.)

93. Schwartz, E. Über zwei Geschwülste bei Kaltblütern. Ztschr. f. Krebsforsch., 1923. 20, 353-357.

94. Ball, H. A. Melanosarcoma and rhabdomyoma in two pine snakes, *Pituophis melanoleucus*. Cancer Res., 1946. 6, 134-138.

95. Stolk, A. Hyperkeratosis and carcinoma planocellulare in the lizard, *Lacerta agilis* L. (preliminary note). Proc. Kon. Ned. Akad. Wet., 1953. 56C, 155-163.

96. Stolk, A. Tumours in reptiles: Melanoma of the skin in the viper *Vipera berus*. Proc. Kon. Ned. Akad. Wet., 1957. 60C, 557-566.

97. Salk, J. E., Youngner, J. S., and Ward, E. N. Using a color change of phenol red as the indicator in titrating poliomyelitis virus or its antibody in a tissue-culture system. Am. J. Hyg., 1954. 60, 214-230.

98. *Manual of Histologic and Special Staining Techniques*. Armed Forces Inst. Pathol., Washington, D. C., 1949.

99. Lentz, T. L., and Trinkaus, J. P. A fine structure study of cytodifferentiation during cleavage, blastula and gastrula stages of *Fundulus heteroclitus*. J. Cell Biol., 1967. 32, 121-138.

100. Bennett, H. S., and Luft, J. H. S-collidine as a basis for buffering fixatives. J. Biophys. Biochem. Cytol., 1959. 6, 113-114.

101. Luft, J. H. Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol., 1961. 9, 409-414.

102. Richardson, K. G., Jarrett, L., and Finke, E. H. Embedding in epoxy resins for ultrathin sectioning in electron microscopy. Stain Technology, 1960. 35, 313-323.

103. Reynolds, E. S. Lead citrate stain. J. Cell Biol., 1963. 17, 208-213.

104. Noyes, W. F. The surface fine structure of vaccinia virus. Virology, 1962. 17, 282-287.

105. Clarke, D. H., and Casals, J. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Am. J. Trop. Med. and Hygiene, 1958. 7, 561-573.

106. Ito, Y. A tumor-producing factor extracted by phenol from papillomatous tissue (Shope) of cottontail rabbits. Virology, 1960. 12, 596-601.

107. Noyes, W. F. Structure of the human wart virus. Virology, 1964. 23, 65-72.

108. Clem, L. W., Moewus, L., and Sigel, M. M. Studies with cells from marine fish in tissue culture. J. Exp. Biol. Med., 1961. 108, 762-766.

109. Wolf, K., and Quimby, M. C. Established eurythermic line of fish cells in vitro. Science, 1962. 135, 1065-1066.

110. Gravell, M., and Milsberger, R. G. A permanent cell line from the fathead minnow (*Pimephales promelas*). Ann. N. Y. Acad. Sci., 1965. 126, 555-565.

111. Gardner, C. E. Studies of Colorado tick fever virus. Unpublished doctoral thesis. Univ. Colorado, 1962.

112. Millonig, G. Further observations on a phosphate buffer for osmium solutions. In S. S. Breese, Jr. (Ed.) Electron Microscopy. Vol. 2 Biology. New York: Academic Press, 1962. pp -8).

113. Palay, S. L. The fine structure of secretory neurons in the preoptic nucleus of the goldfish *(Carassius auratus)*. Anat. Res., 1960. 138, 417-443.

114. Trump, B. F., and Bulger, R. E. New ultrastructural characteristics of cells fixed in a glutaraldehyde-osmium tetroxide mixture. Lab. Invest., 1966. 15, 368-379.

115. Karnovsky, M. J. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J. Cell Biol., 1965. 27, 137a-138a.

116. Zelickson, A. S. Ultrastructure of normal and abnormal skin. Philadelphia: Lea and Fiebiger, 1967. pp 228-260.

117. Setoguti, T. Ultrastructure of guanophores. J. Ultrastruct. Res., 196u. 18, 324-332.

118. Matsumoto, J. Studies on fine structure and cytochemical properties of erythrophores in swordtail, *Xiphophorus helleri*, with special reference to their pigment granules (pterinosomes). J. Cell Biol., 1965. 27, 493-504.

119. Schwerdt, C. E., and Schaffer, F. L. Some physical and chemical properties of purified poliomyelitis virus preparations. Ann. N. Y. Acad. Sci., 1955. 61, 740-753.

120. Morgan, C., Howe, C., and Rose, H. M. Intercellular crystals of coxsackie virus viewed in the electron microscope. Virology, 1959. 9, 145-149.

121. Willis, R. A. Pathology of tumours. (2nd Ed.) St. Louis, Mo.: C. V. Mosby Co., 1953. pp 1-8.

122. Orcutt, H. G. The life history of the starry flounder, *Platichthys stellatus* (Pallas). Calif. Fish and Game, 1950. Fish Bull. 78, 7-64.

123. Walker, R., and Weissenberg, R. Conformity of light and electron microscopic studies on virus particle distribution in lymphocystis tumor cells of fish. Ann. N. Y. Acad. Sci., 1965. 126, 375-385.

124. Huxley, J. Biological aspects of cancer. London: Allen and Unwin, 1958. pp 18-35.

125. Balls, M. Spontaneous neoplasms in amphibia: A review and descriptions of six new cases. Cancer Res., 1962. 22, 1142-1154.

126. Schäperclaus, W. Fischkrankheiten. Braunschweig: G. Wenzel and Sohn, 1935. (Cited in Nigrelli, 1952.)

127. Wolf, L. E. Dietary disease of trout. Fisheries Res. Bull., 1945. 7, 1-30.

128. Loewenthal, W. Einschlüssartige Zell- und Kernveränderungen in der Karpfenpocke. Ztschr. f. Krebsforsch., 1907. 5, 197. (Cited in Nigrelli, 1952.

LIST OF ILLUSTRATIONS

ABBREVIATIONS FOR ILLUSTRATIONS

BM:	Basement membrane
BV:	Blood vessel
C_1:	Basal cell
C_2-C_4:	Intermediate cell
C_5:	Surface cell
CG:	Cytoplasmic granule
CM:	Cell membrane
DC:	Dermal collagen
EP:	Erythrophore
FB:	Fibroblast
IR:	Iridocyte
IC:	Intercellular space
J:	Junctional complex
M:	Mucous cell
N:	Nucleus
NC:	Nucleolus
N_1:	Basal cell nucleus
N_2-N_4:	Intermediate cell nucleus
N_5:	Surface cell nucleus
NC:	Nucleolus
NP:	Nuclear pore
MN:	Mucous cell nucleus
UC:	Unknown cell
V:	Vacuole

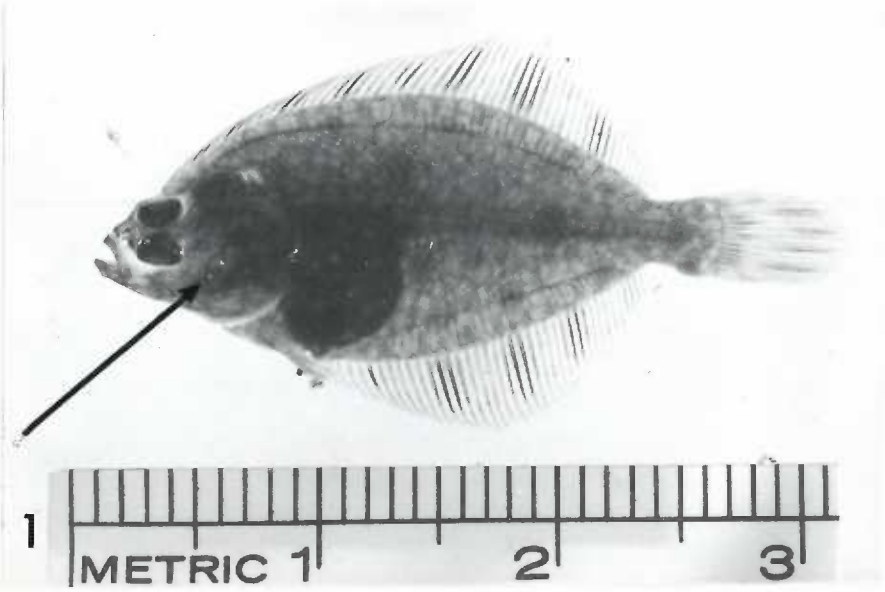
VP:	Virus particle
cf:	Cytoplasmic filaments
er:	Ergastoplasm
d:	Desmosome
g:	Golgi apparatus
m:	Mitochondrion
mp:	Membrane-bound particle
mv:	Microvillus
r:	Ribosome
ss:	Subdermal space
v:	vesicle

Figure 1:

Early postmetamorphosis starry flounder, age group 0, with angio-epithelial nodules on the pigmented side of the fish.

Figure 2:

Early postmetamorphosis starry flounder, age group 0, with angio-epithelial nodules on the pigmented side of the fish.


1
METRIC 1
2
3

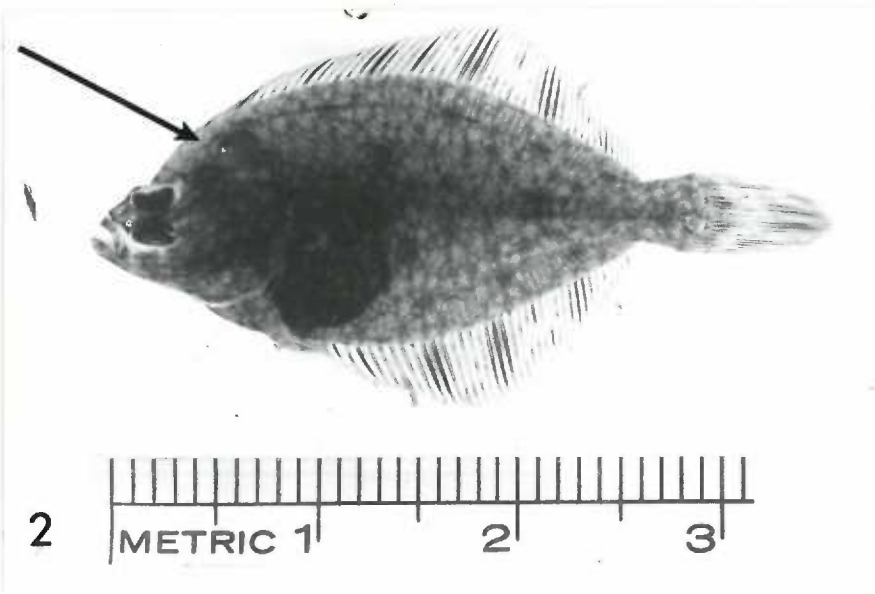
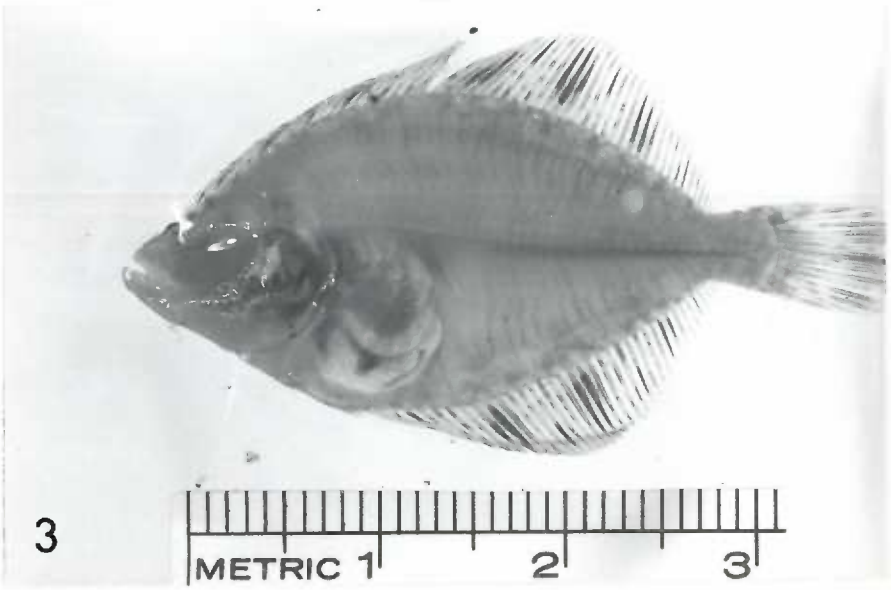

2
METRIC 1
2
3

Figure 3:

Early postmetamorphosis starry flounder, age group 0, with an angioepithelial nodule on the nonpigmented side of the fish.

Figure 4:

Early postmetamorphosis starry flounder, age group 0, with an angioepithelial nodule on the nonpigmented side of the fish.


3
METRIC 1
2
3

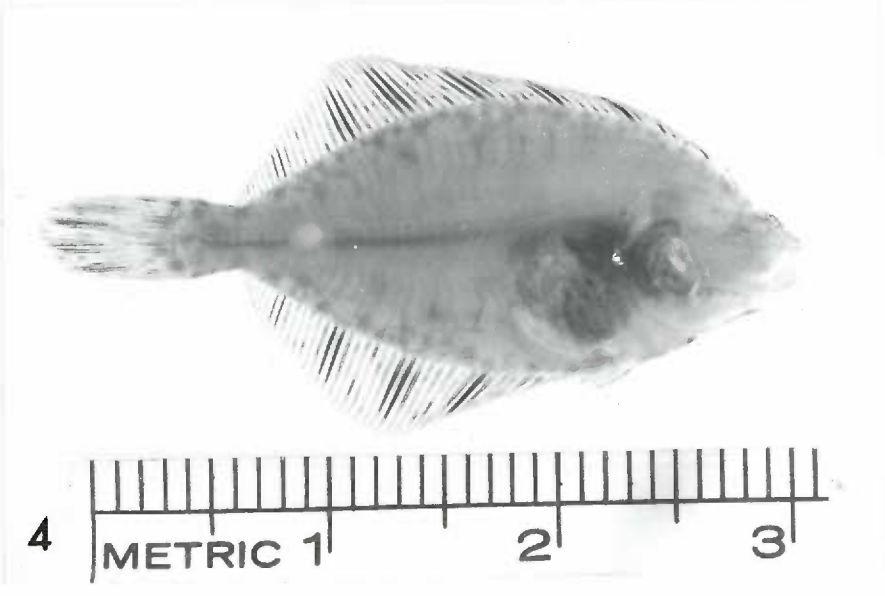
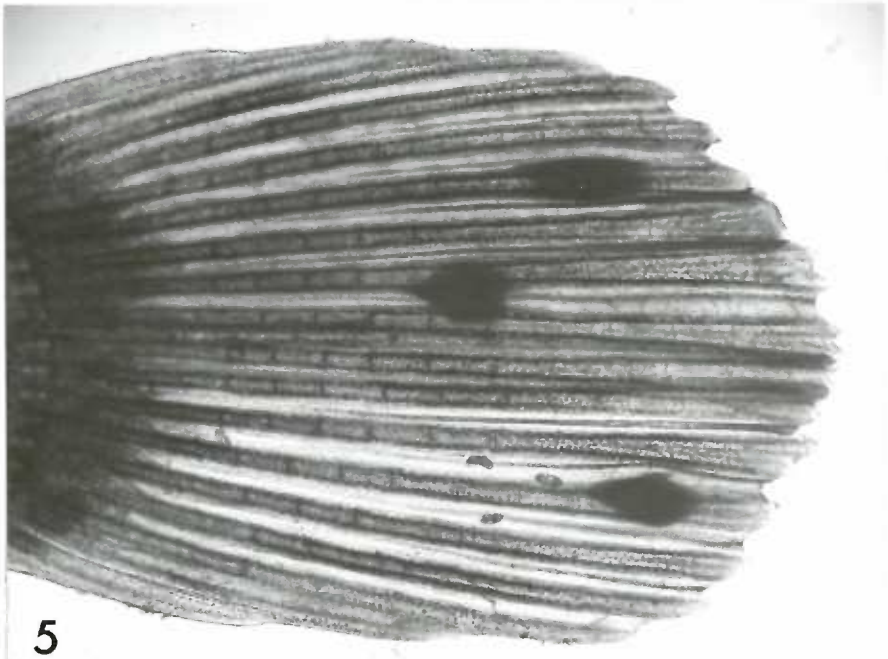

4
METRIC 1
2
3

Figure 5:

Tail region of an early postmetamorphosis starry flounder showing three angioepithelial nodules. Note the three trematodes located in the area of the tumors.



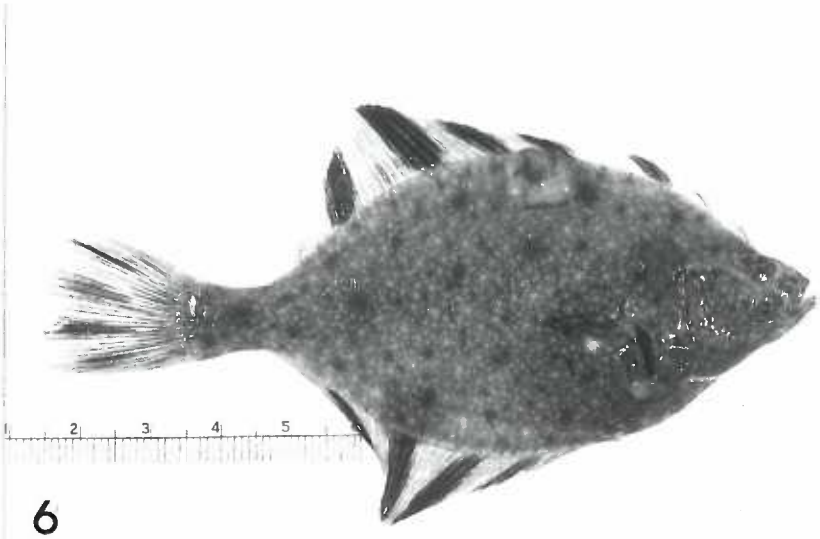
5

Figure 6:

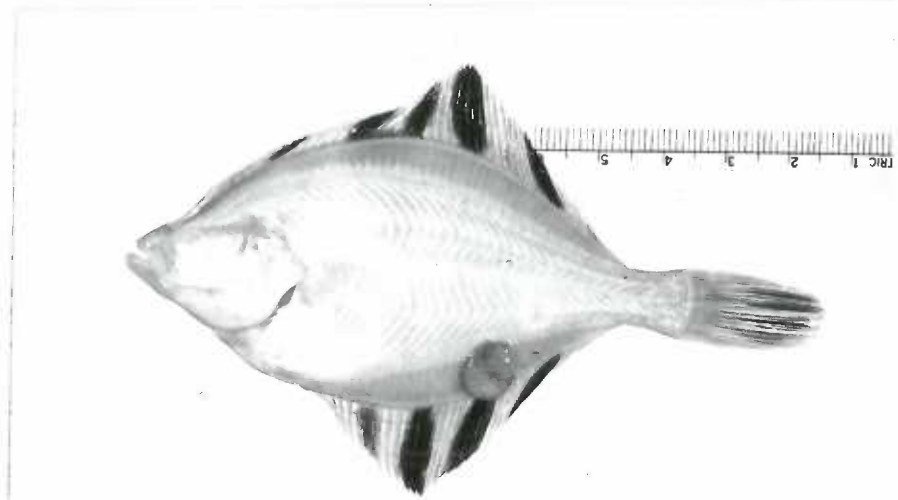
Starry flounder, age group I, with three papillomas on the pigmented side, located in quadrants 1 and 2.

Figure 7:

Starry flounder, same fish as Figure 6, with a papilloma on the nonpigmented side, located in quadrant 7.



6



7

Figure 8:

Starry flounder, age group I, with a single pigmented papilloma on the nonpigmented side, located in quadrant 5.


8
METRIC 1
2
3
4
5

Figure 9:

Starry flounder, age group I, with a papilloma in quadrant 1 and another on the tail on the pigmented side of the fish.

Figure 10:

A closer view of the papilloma seen in Figure 9. Note the typical papillomatous morphology of the tumor.

9

10

METRIC 1 2 3 4

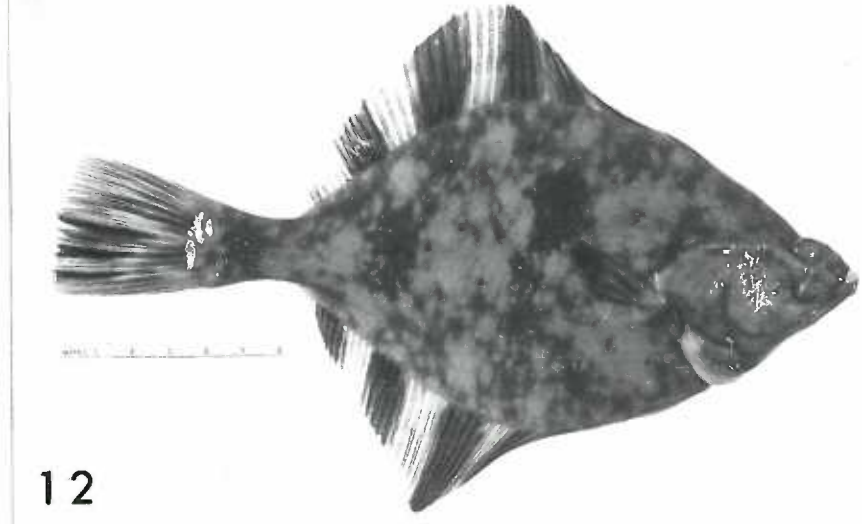
Figure 11:

Starry flounder, age group I, with papillomas on the tail and dorsal fin. These two lesions involved both the pigmented and non-pigmented sides of the specimen.

Figure 12:

Starry flounder, age group II, with a papilloma on the pigmented side, located on the gills of the specimen.

11



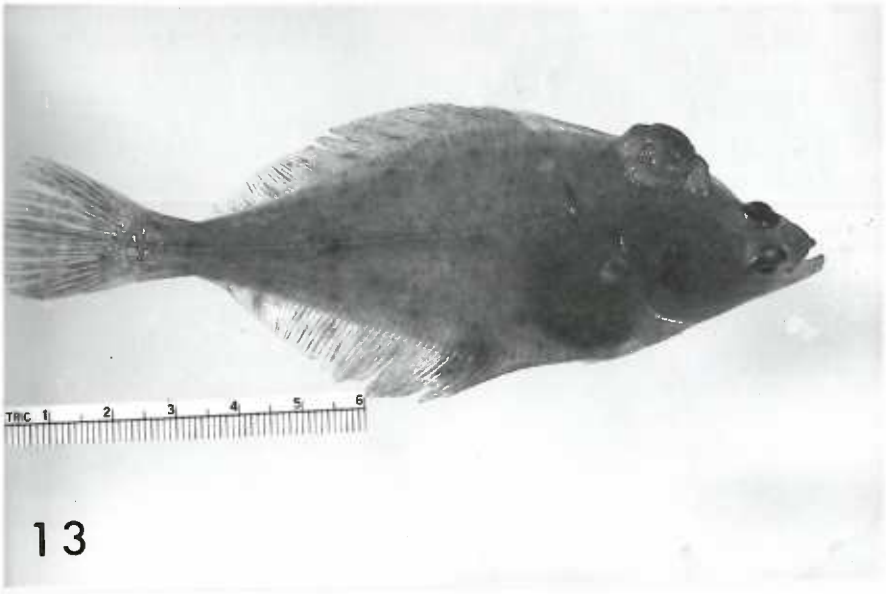
12

Figure 13:

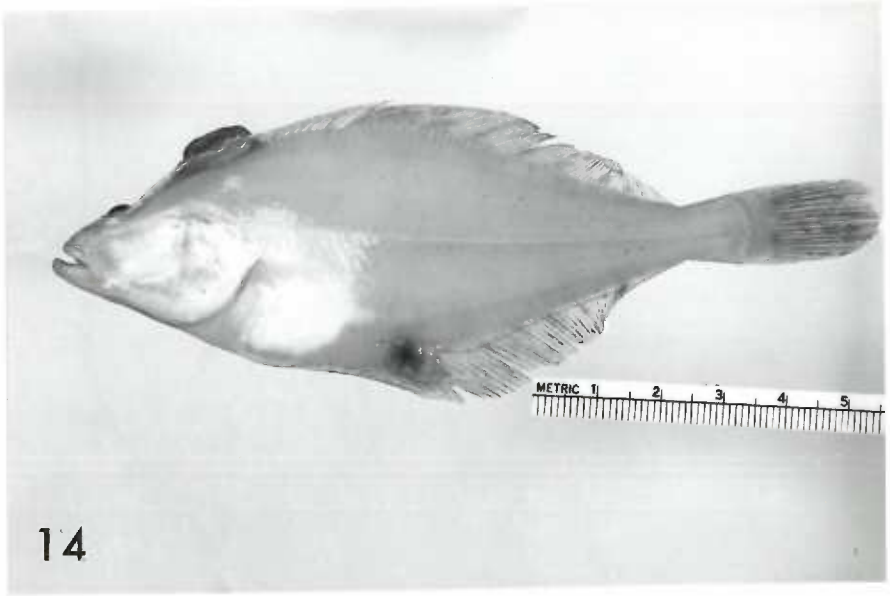
English sole, age group I, with a large papilloma on the pigmented side of the fish.

Figure 14:

English sole with a papilloma on the nonpigmented side of the fish seen in Figure 13. Note the papilloma on the pigmented side has apparently grown through and involved the nonpigmented side.



13



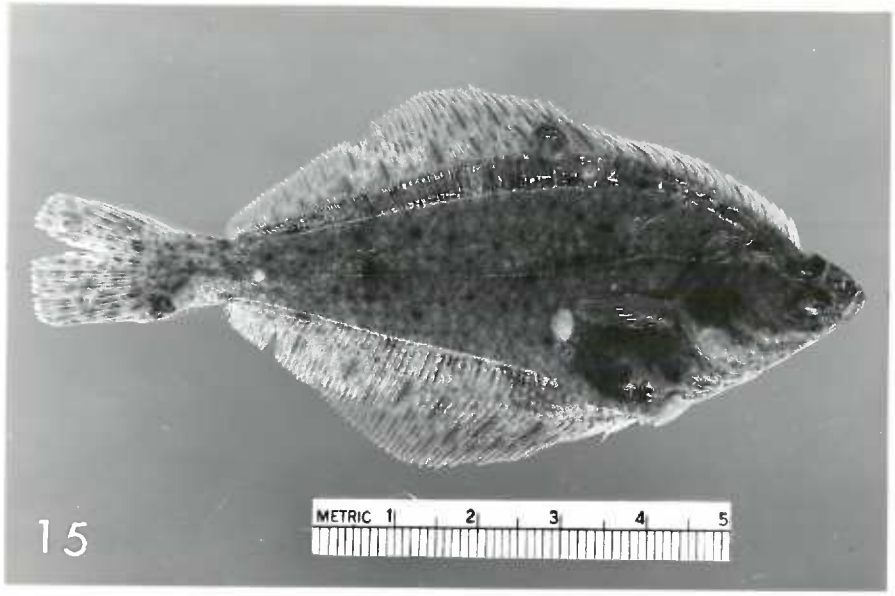
14

Figure 15:

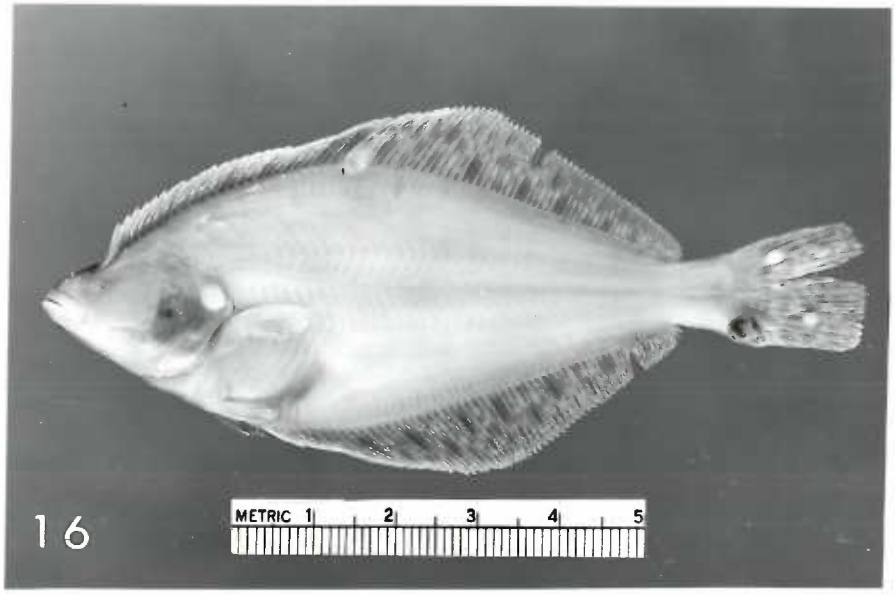
English sole, age group 0, with numerous pigmented and nonpigmented angioepithelial nodules on the pigmented side.

Figure 16:

English sole, the nonpigmented side of the fish seen in Figure 15 showing numerous nonpigmented and one pigmented angioepithelial nodules.



15



16

Figure 17:

English sole, age group I, with a large papilloma on the dorsal fin and a large papilloma almost engulfing the head, on the pigmented side.

Figure 18:

English sole, age group III, with multiple papillomas, one involving the right pectoral fin. Note the flat pigmented lesion involving the whole anterior portion of the fish.


17
METRIC


18
METRIC

Figure 19:

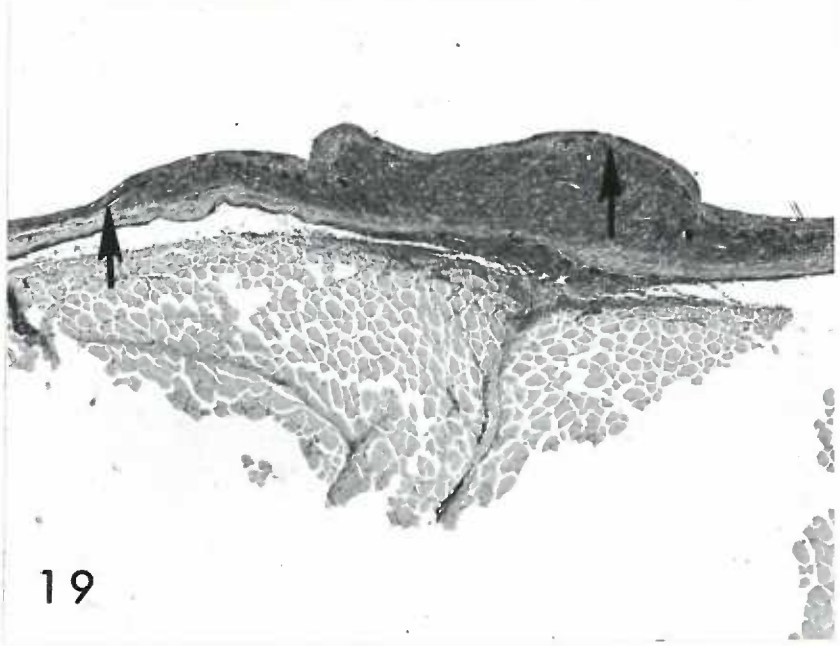
Low power micrograph of an angioepithelial nodule from a starry flounder. The lesion is bordered by normal skin. The lesion consists mostly of vascular connective tissue capped by a layer of epidermis. Arrows indicate the location of the dermal-epidermal junction.

Hematoxylin and eosin stain. 65X.

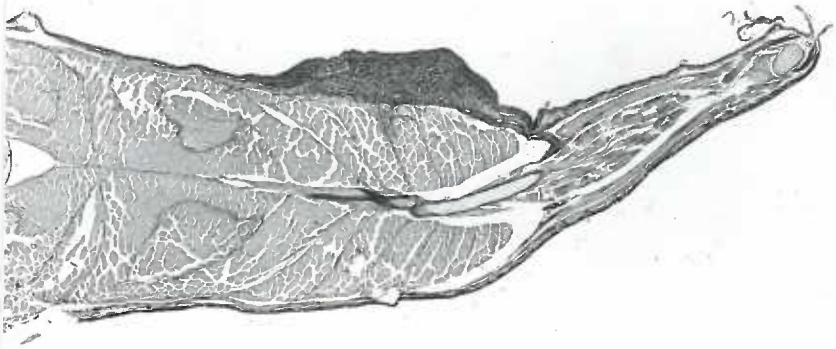
Figure 20:

Low power micrograph of an angioepithelial nodule from a starry flounder. Similar in morphology to the lesion seen in Figure 19.

Hematoxylin and eosin stain. 31X.



19



20

Figure 21:

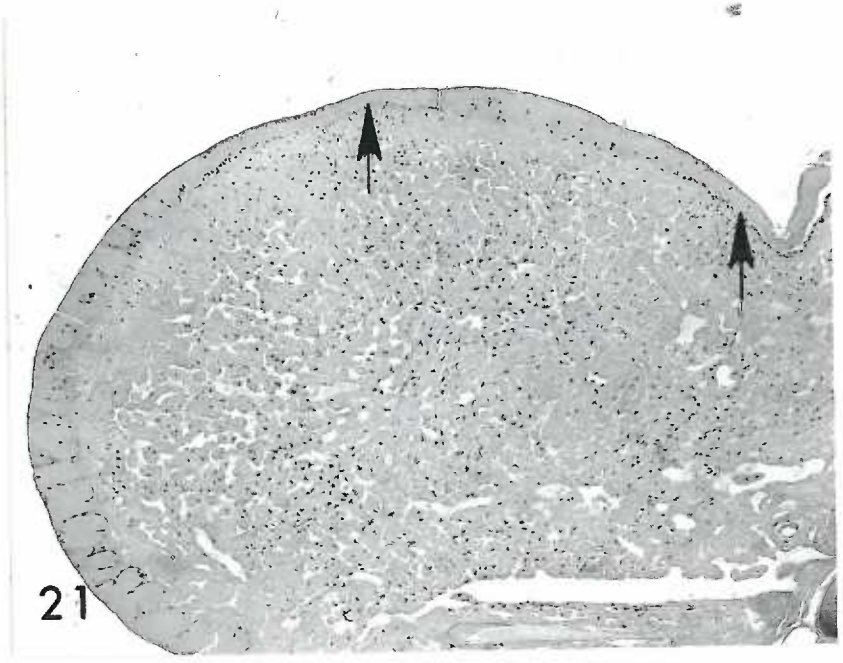
Low power micrograph of an angioepithelial nodule from an English sole. The lesion contains mostly vascular connective tissue capped by a layer of mildly hyperplastic epidermis. Mucous cells are decreased and small dense black spots represent melanophores located in the dermis and epidermis. Arrows indicate the dermal-epidermal junction.

Hematoxylin and eosin stain. 33X.

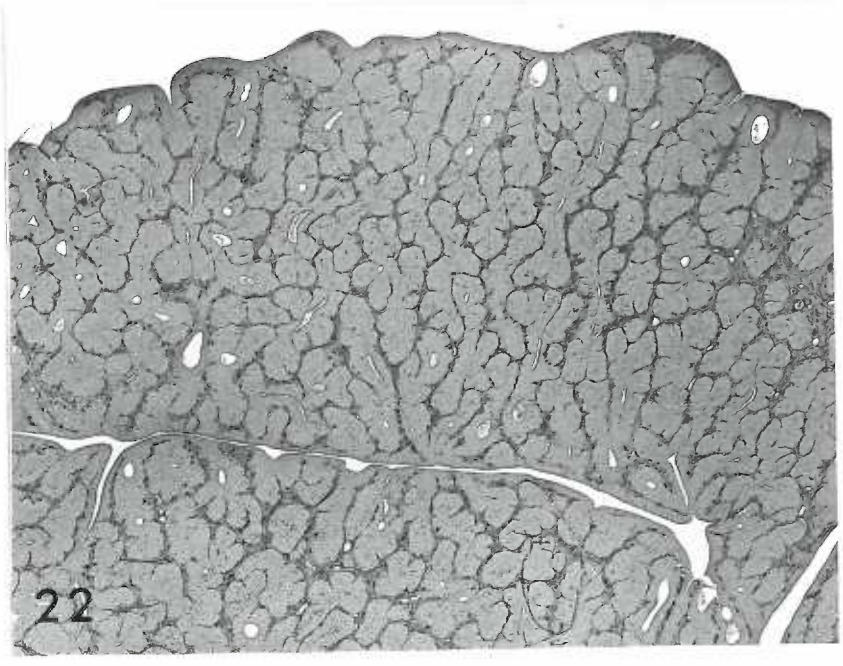
Figure 22:

Low power micrograph of an epidermal papilloma from an English sole. The darkly staining collagenous stroma supports the thick layers of epidermal cells.

Hematoxylin and eosin stain. 22X.



21



22

Figure 23:

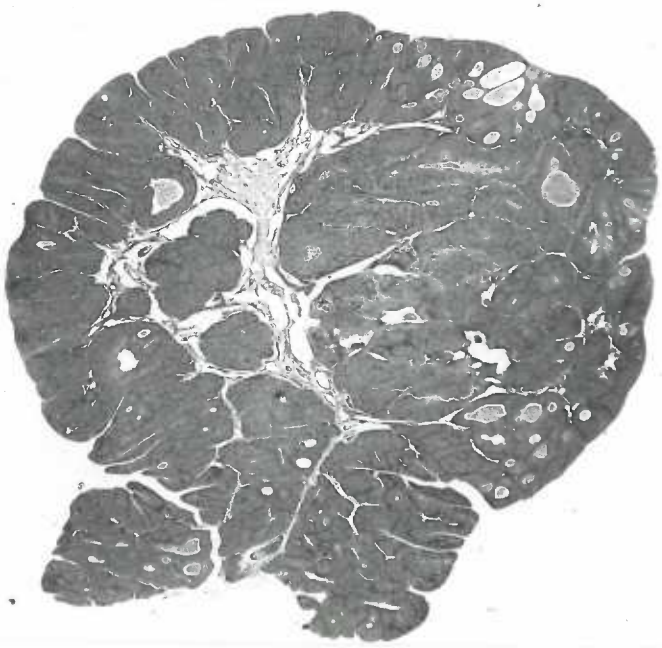
Lower power micrograph of an epidermal papilloma from an English sole. Note the dense central connective tissue core which branches and supports the thick epidermis which has been thrown into many folds.

Hematoxylin and eosin stain. 12X.

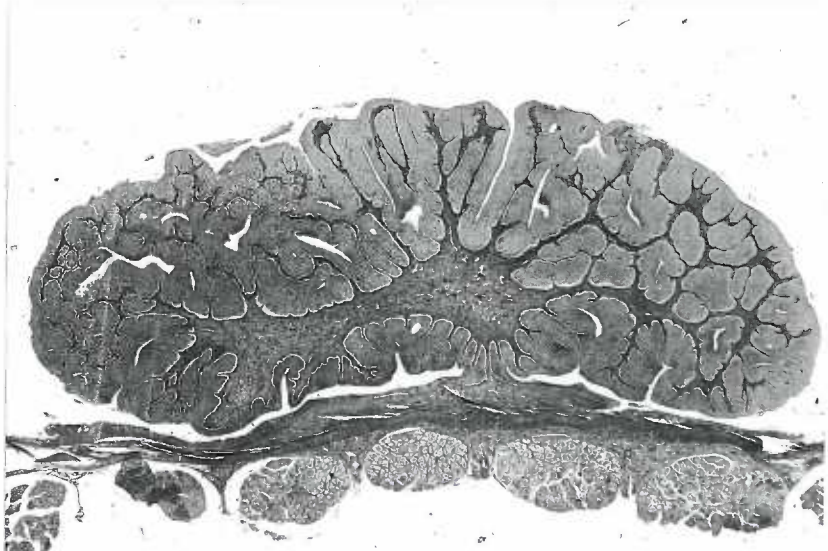
Figure 24:

Low power micrograph of an angioepithelial polyp from a starry flounder showing the distribution of the stroma. This lesion shows hyperplasia of the epidermis. Stromal components are seen branching and supporting epithelial folds. Note the stalk at the base of the lesion. Directly below the stalk, dermal involvement is seen with little or no epidermal involvement lateral to the base of the stalk.

Reticulum stain. 19X.



23.



24

Figure 25:

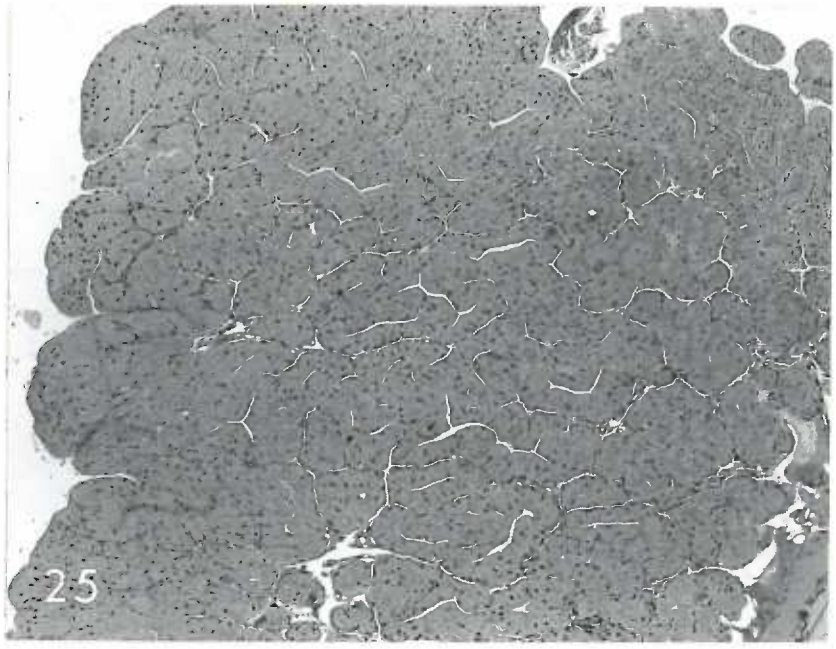
Low power micrograph of an epidermal papilloma from a starry flounder. Note the large number of melanophores associated with the stromal component of the lesion.

Hematoxylin and eosin stain. 31X.

Figure 26:

High power micrograph of an epidermal papilloma from an English sole. The stroma (ST) contains black melanin granules, identifying the location of melanophores. The basement membrane separates the dermis from the epidermis. Note the large cell in the epidermis, which contains a prominent nucleolus within a well-defined nucleus.

Epon embedded, 1 μ section, stained with toluidine blue. 575X.


25

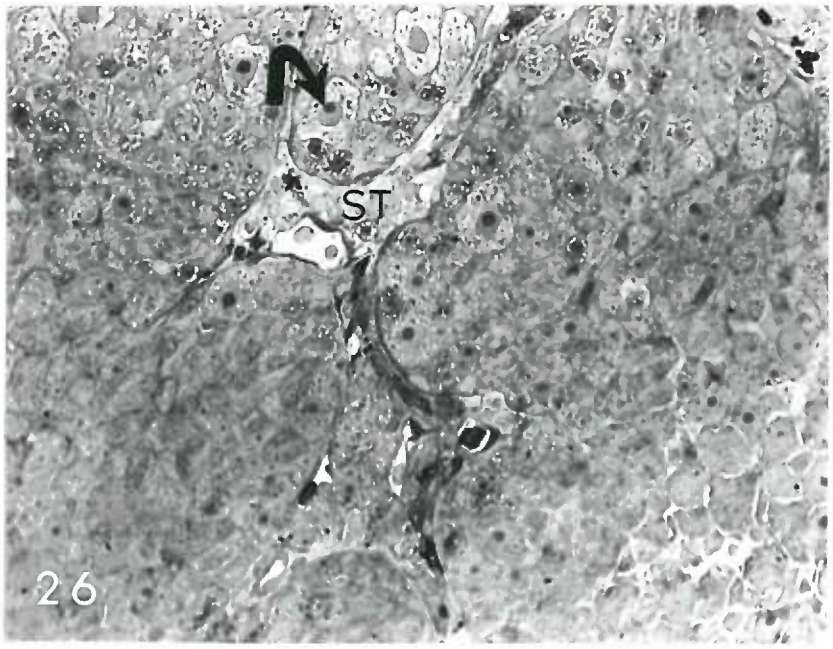

N
ST
26

Figure 27:

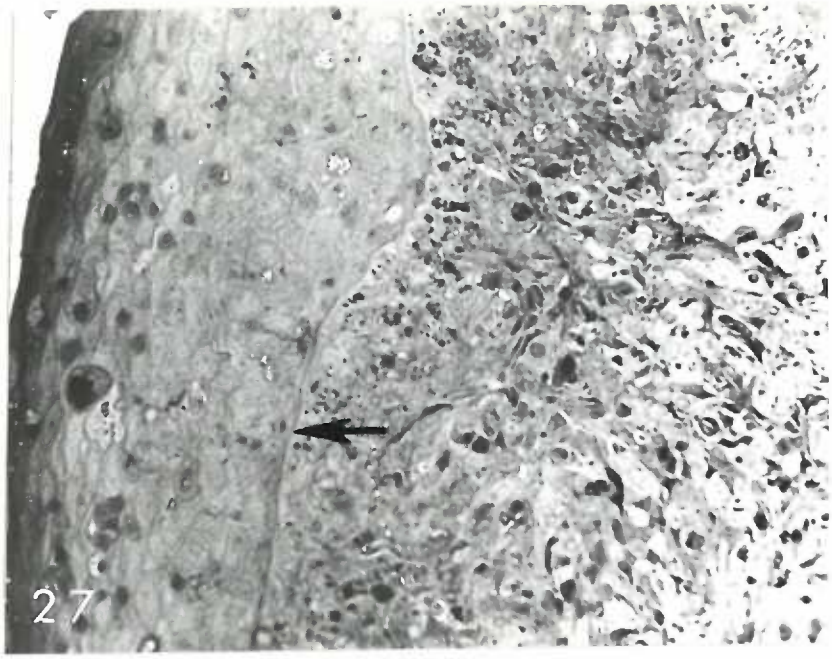
High power micrograph of the angioepithelial polyp seen in Figure 24, showing the epidermis (left) and the dermis (right). The arrow marks the epidermal-dermal interface. The epidermis is well-differentiated and contains mucous cells.

Epon embedded, 1 μ section, stained with toluidine blue. 480X.

Figure 28:

High power light micrograph of an angioepithelial nodule of an English sole, illustrating the epidermis (left) and the dermis (right). Arrows indicate dermal-epidermal junction. Unidentified small cells with dark nuclei may be inflammatory.

Epon embedded, 1 μ section, stained with toluidine blue. 575X.


27

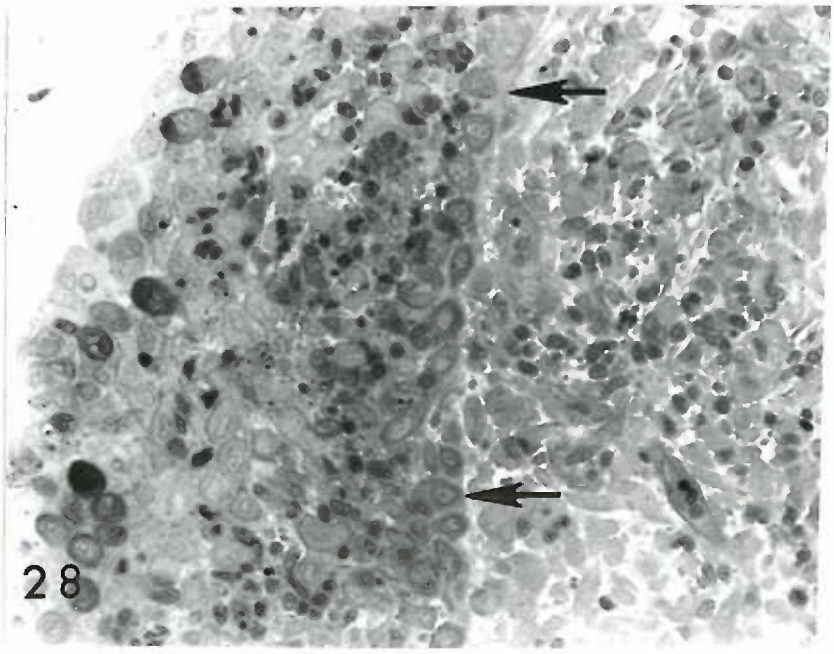

28

Figure 29:

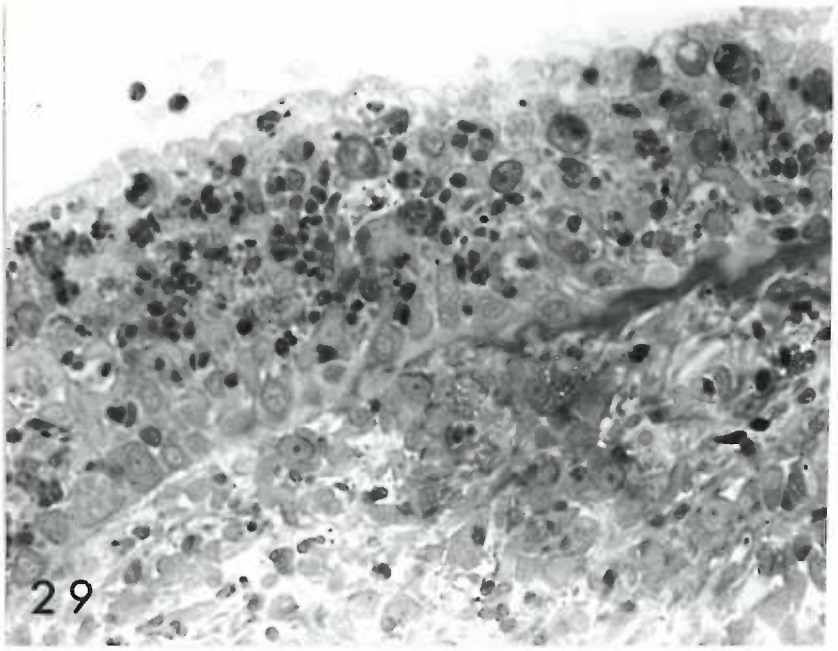
Same as Figure 28, starry flounder angioepithelial nodule. Arrows indicate the location of the dermal-epidermal interface.

Epon embedded, 1 μ section, stained with toluidine blue. 575X.

Figure 30:

High power micrograph of an epidermal papilloma from a starry flounder. The prominent basement membrane separates the stroma (ST) from the epithelial components. The arrow indicates the nuclear membrane of an epithelial cell. This nucleus contains a nucleolus. Note that there are cells in the stroma with dark granules which resemble cells in the epidermis.

Epon embedded, 1 μ section, stained with toluidine blue. 575X.


29

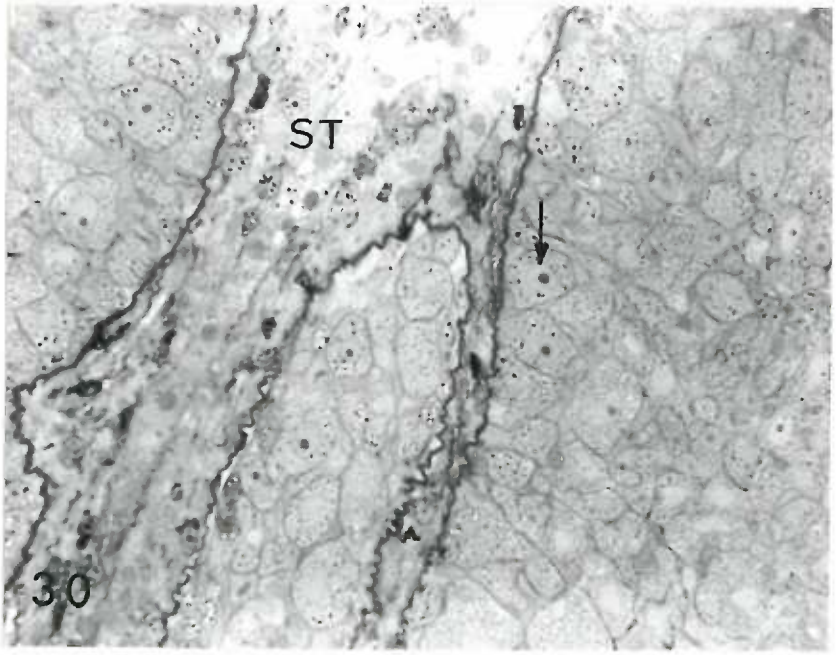

ST
30

Figure 31:

Normal larval epidermis. Cell layers in fin fold epithelium. The superficial surface cell (N_2) has microvilli (mv), ergastoplasm (er), and a Golgi apparatus. Cytoplasmic filaments (cf) are limited to the area directly below the cytoplasmic membrane of the superficial cell. These filaments are not found in the deep epidermal layer (N_1). Directly below the deep layer lies a faint line considered to be the primitive basement membrane (see arrows). A subdermal space (SS) contains a particulate material and separates the epidermis from the mesoderm (MC). Dermal components are apparently absent at this stage of development.

Lead citrate, uranyl acetate stain. 20,900X.

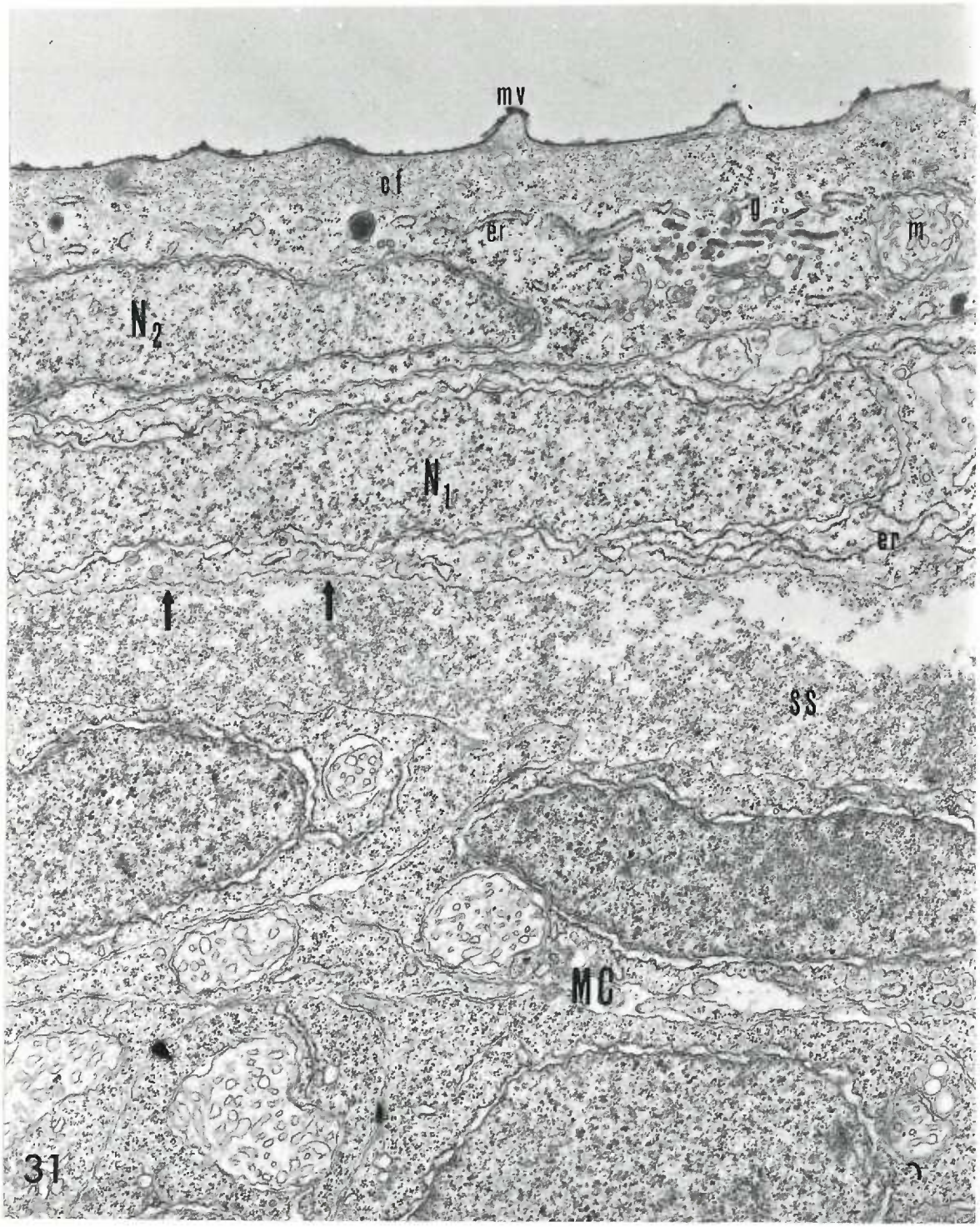

mv
cf
er
g
m
N2
N1
er
SS
MC
31

Figure 32:

Normal larval epidermis. Cell layers of fin fold epithelium. Two cells are adjoined at their superficial surface by a junctional complex (J) of a tight junction (zonula occludens) and one or more desmosomal (d) interconnections. The cytoplasm of one cell extends across the electron micrograph and contains mitochondria (m) and ergastoplasm (er). The arrow indicates free ribosome-like cytoplasmic particles. The primitive basement membrane (BM) divides the epidermis from the subdermal space (ss).

Lead citrate, uranyl acetate stain. 32,800X.

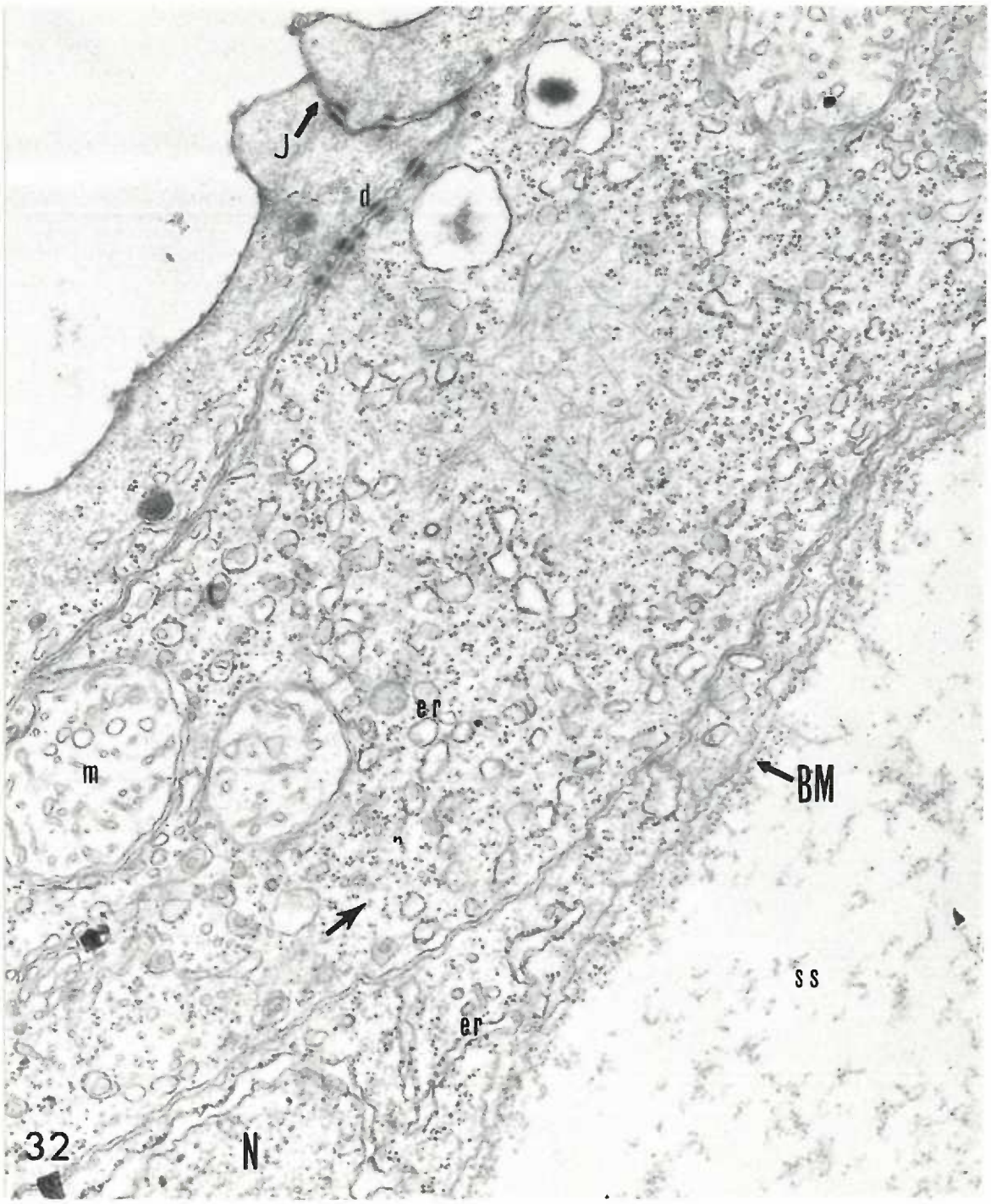
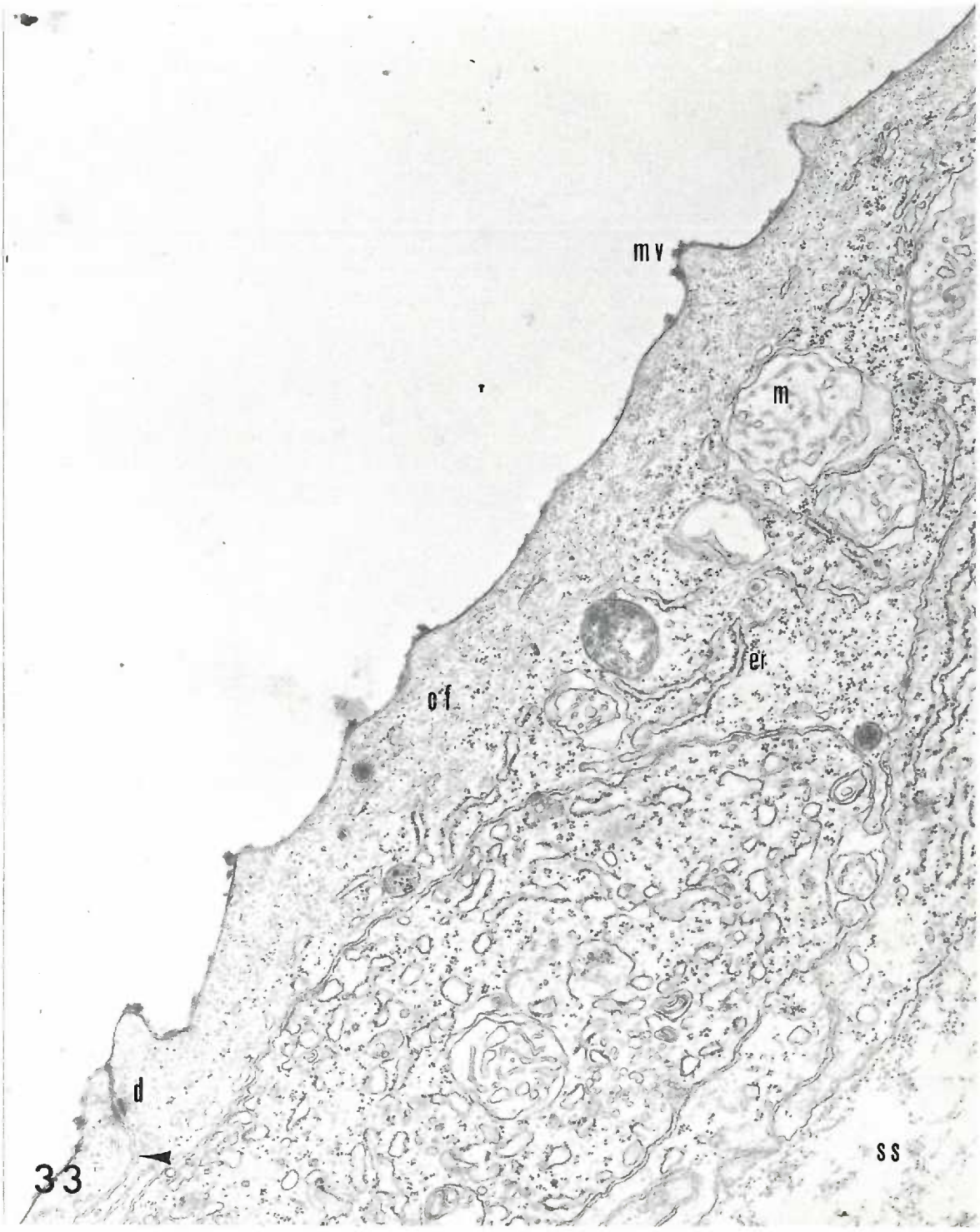

J
d
er
m
BM
ss
er
32
N

Figure 33:

Normal larval epidermis. Cells of fin fold epithelium. Superficial cell contains microvilli (mv), cytoplasmic filaments (cf), mitochondria (m), and ergastoplasm. The arrow indicates a junctional complex and interdigitating cytoplasmic membranes.

Lead citrate, uranyl acetate stain. 20,900X.



33

Figure 34:

An electron micrograph of the full thickness of normal age group 0 epidermis and the dermis. Basal cells (C_1) exhibit marked interdigitation and desmosomal intercellular connections with adjacent cells. Numerous mitochondria and a well-developed ergastoplasm. Intermediate cells (C_2 and C_3) show numerous intracellular interconnections. Their ergastoplasm is well developed and mitochondria are numerous. The density of the matrix in the intermediate cells varies. The superficial cell contains many microvilli. The basement membrane (BM) is well formed as is the dermal collagen (DC). The arrow indicates a blood vessel in the dermis. Note the large number of dermal collagen layers.

Lead citrate, uranyl acetate stain. 13,640X.

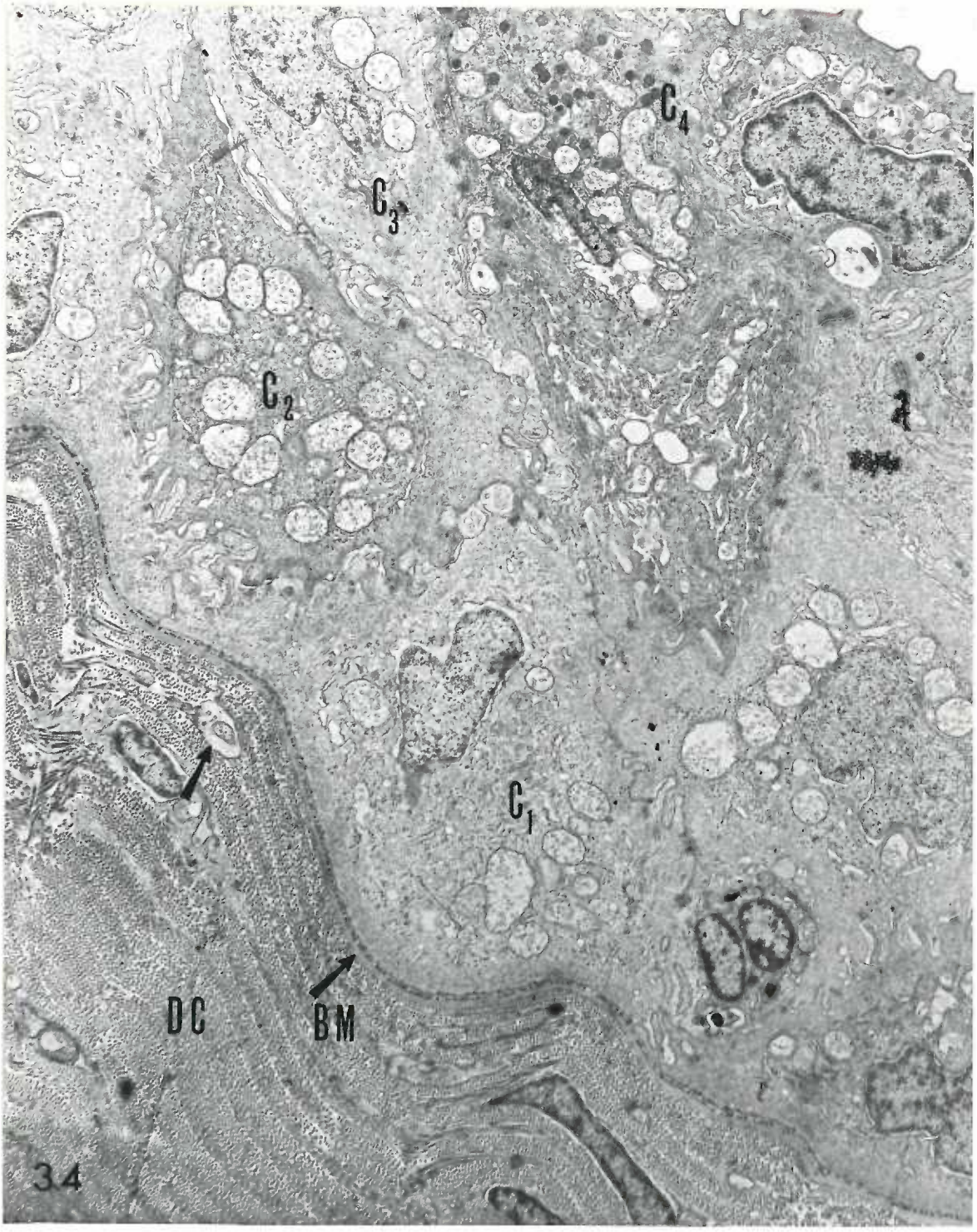

C4
C3
C2
C1
DC
BM
3.4

Figure 35:

An electron micrograph of normal age group 0 epidermis. The superficial cell (C_4) contains a well-developed ergastoplasm, contains many mitochondria (m). Desmosomes (d) and membrane interdigitation of adjacent cells are obvious. Note the density of the cytoplasmic matrix.

Lead citrate, uranyl acetate stain. 17,400X.

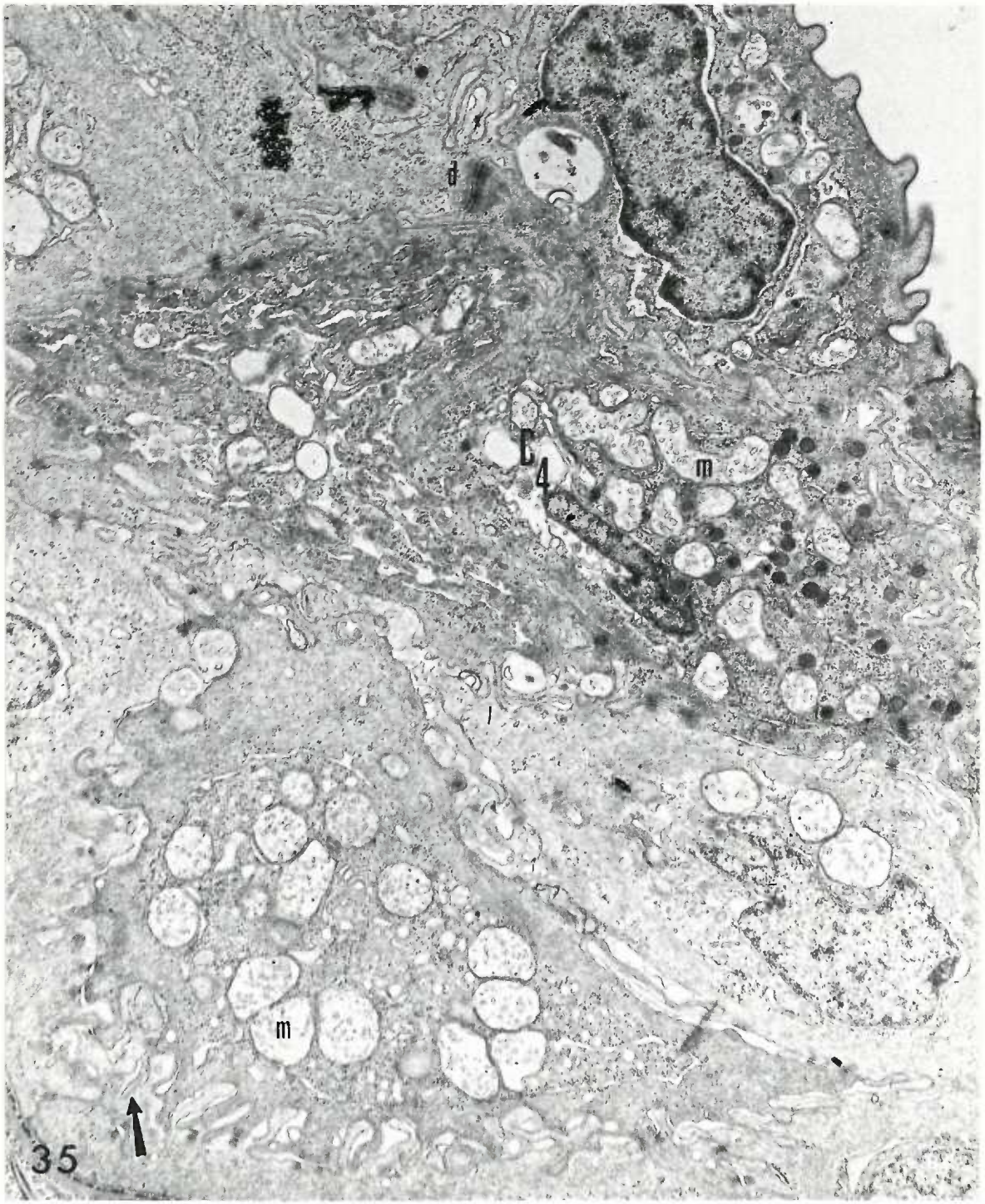

d
C4
m
l
m
35

Figure 36:

A higher magnification of Figure 34, showing superficial cells with large numbers of cytoplasmic filaments (cf) and membrane interdigitations (see arrows). A large structure staining similar to nuclear material is seen. Distributed throughout the cytoplasm of the surface cells are dense membrane-bound particles. The surface contains many microvilli (mv) which are covered by an amorphous mass. Mitochondria (m) are numerous, as are desmosomes (d). A Golgi apparatus (g) is seen with its typical flattened sacs.

Lead citrate, uranyl acetate stain. 20,500X.

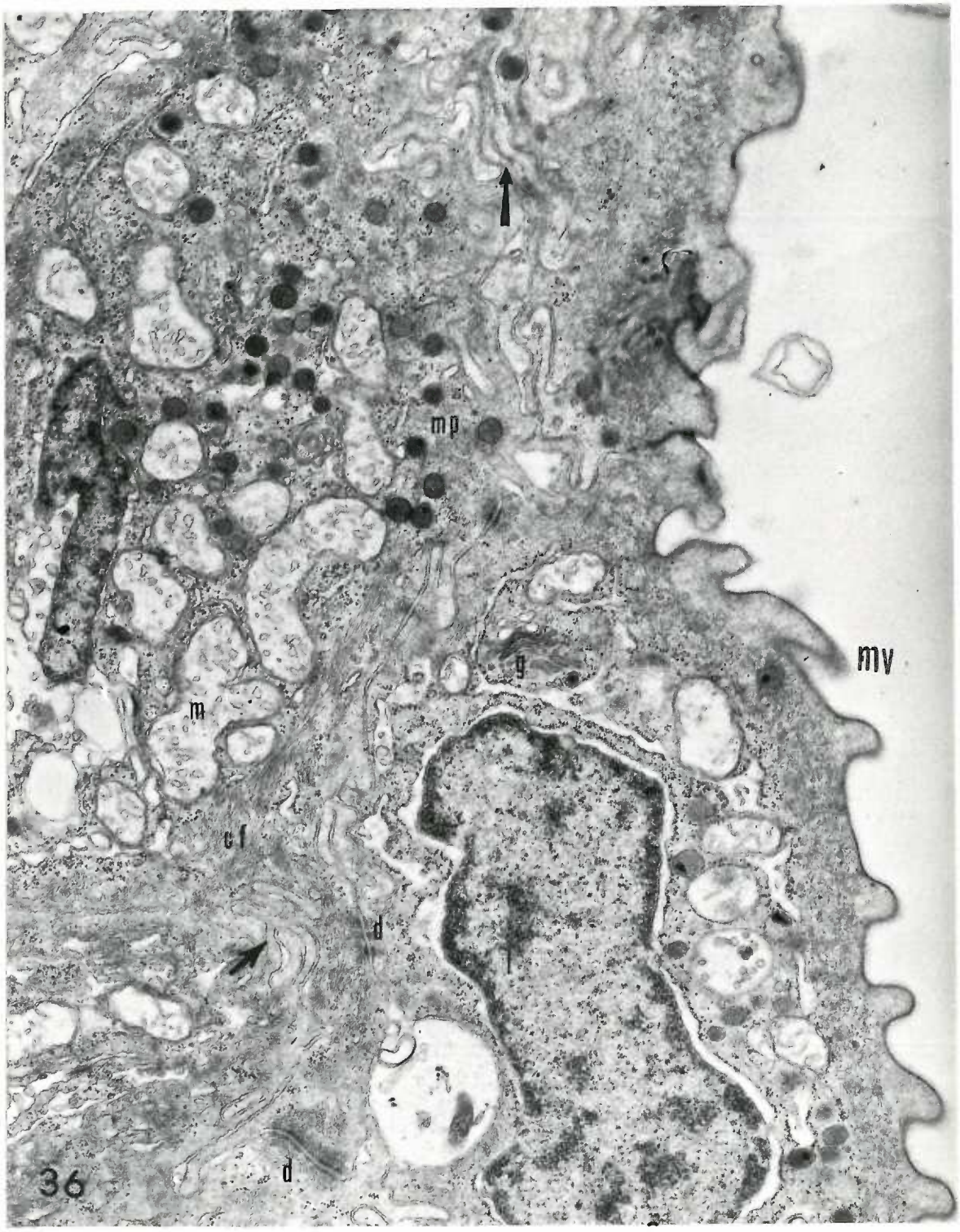

mp
mv
g
m
cf
d
d
36

Figure 37:

An electron micrograph of full thickness of normal age group I epidermis and dermis. The subcellular complement of the epidermal cells is like that of age group 0. The number of cell layers has increased. Cell variety and cell size differences are marked. The arrow indicates the basement membrane at the epidermal-dermal junction. Dermal collagen (DC) is well organized.

Lead citrate, uranyl acetate stain. 10,500X.

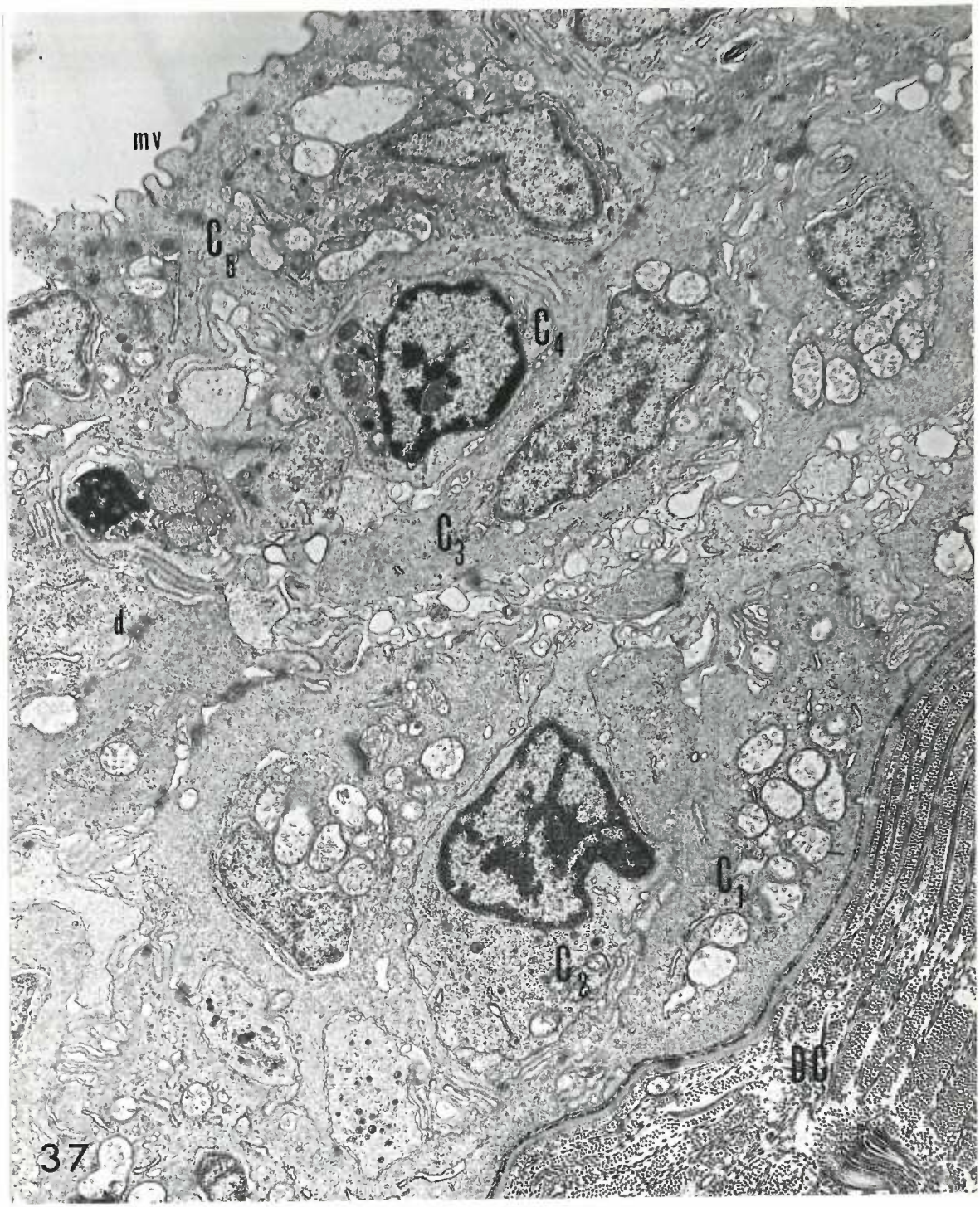

mv
C_8
C_4
C_3
d
C_1
C_2
DC
37

Figure 38:

An electron micrograph of the basal and intermediate cell layers of normal age group I epidermis. The dermal collagen (DC) is separated from the epidermis by a slightly dense basement membrane (BM). The arrow indicates a blood vessel in the dermis. Three cell layers are seen in this micrograph: the basal cell layer (C_1) and two intermediate cell layers (C_2, C_3). The basal cell (C_1) shows many mitochondria (m) and occasional desmosomes connect these cells with adjacent cells. Intermediate C_2 exhibits a central cellular structure which contains mitochondria, a Golgi apparatus (g), ergastoplasm, and a nuclear structure showing marginated chromatin. The central area of C_2 is quite different in texture from the surrounding cytoplasm, which contains many cytoplasmic filaments. Desmosomes and cytoplasmic filaments are observed throughout the cytoplasm of the intermediate cells.

Lead citrate, uranyl acetate stain. 14,500X.

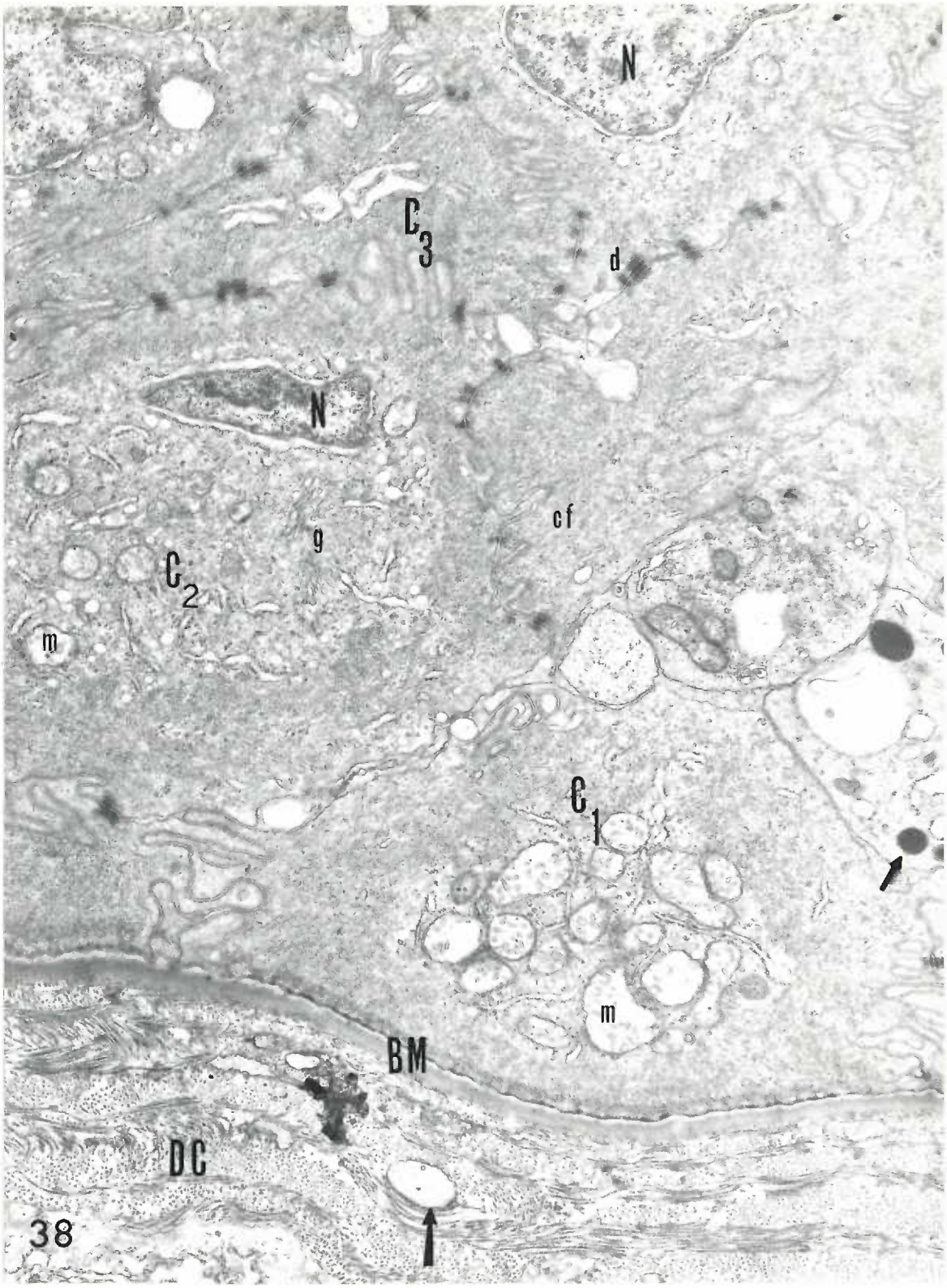

N
C3
d
N
cf
g
C2
m
C1
m
BM
DC
38

Figure 39:

An electron micrograph showing two unknown cells in the epidermis which are different from surrounding cells. The upper cell contains mitochondria (m) and ergastoplasm and a particulate cytoplasm (see arrow). The cell beneath it has a less dense matrix and contains many densely staining cytoplasmic inclusions. These two cells occur at the junction of intermediate and basal cells, and apparently does not form interconnections with these cells.

Lead citrate, uranyl acetate stain. 20,900X.

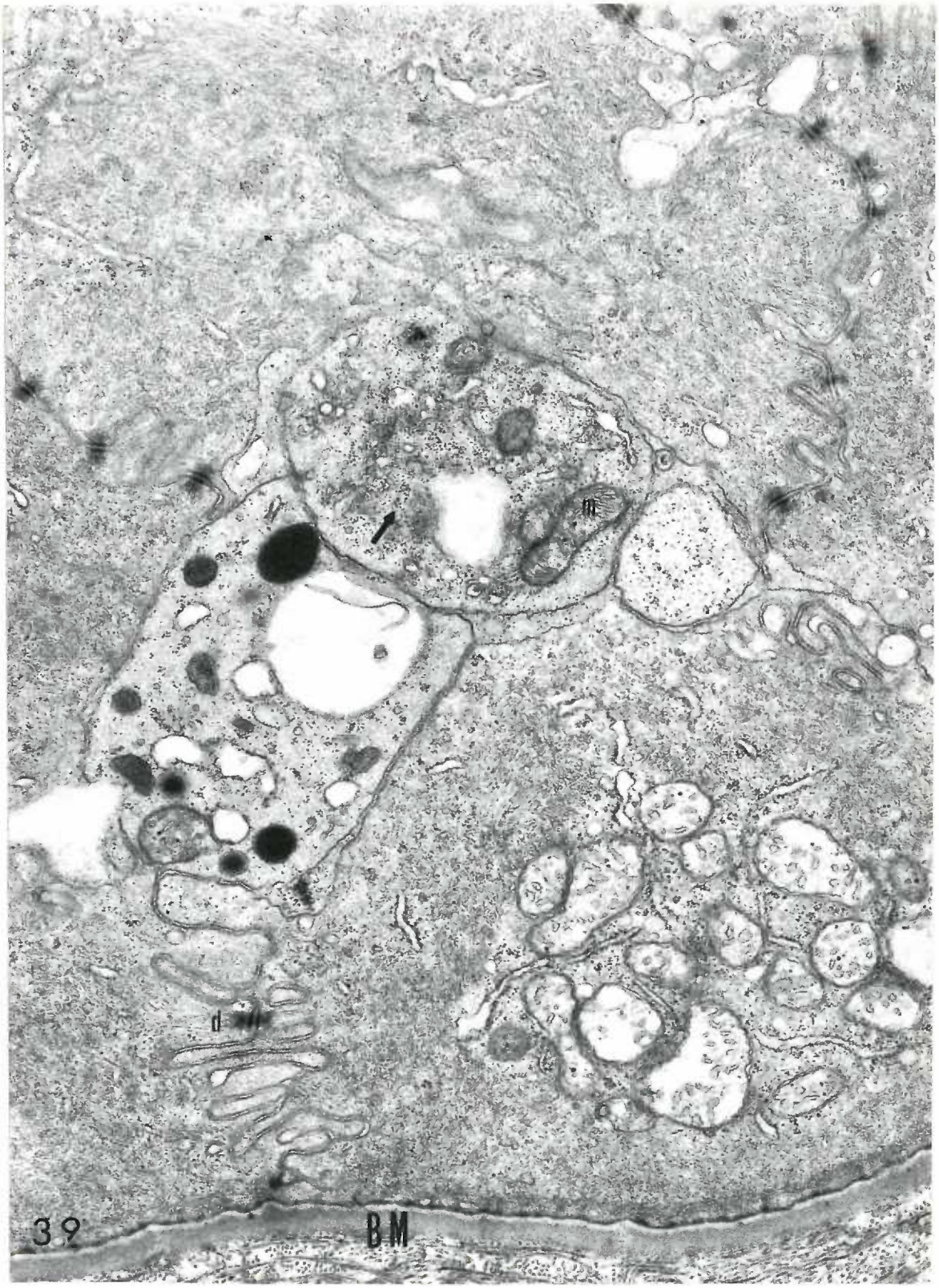

m
d
39
BM

Figure 40:

An electron micrograph of the intermediate layer of normal age group I epidermis. There is marked variation in the size and morphology of the nuclei at the various levels of the epidermis. The nucleus (N_1) is very irregular in shape and exhibits inversion of its structure by cytoplasmic elements. Nuclei N_2 and N_3 show marked margination of their chromatin, quite different from the surrounding cells. In the nucleus of N_5 a dense structure is seen which may or may not be a nucleolus. Many desmosomes and intercellular connections are seen at the level of the epidermis.

Lead citrate, uranyl acetate stain. 14,200X.

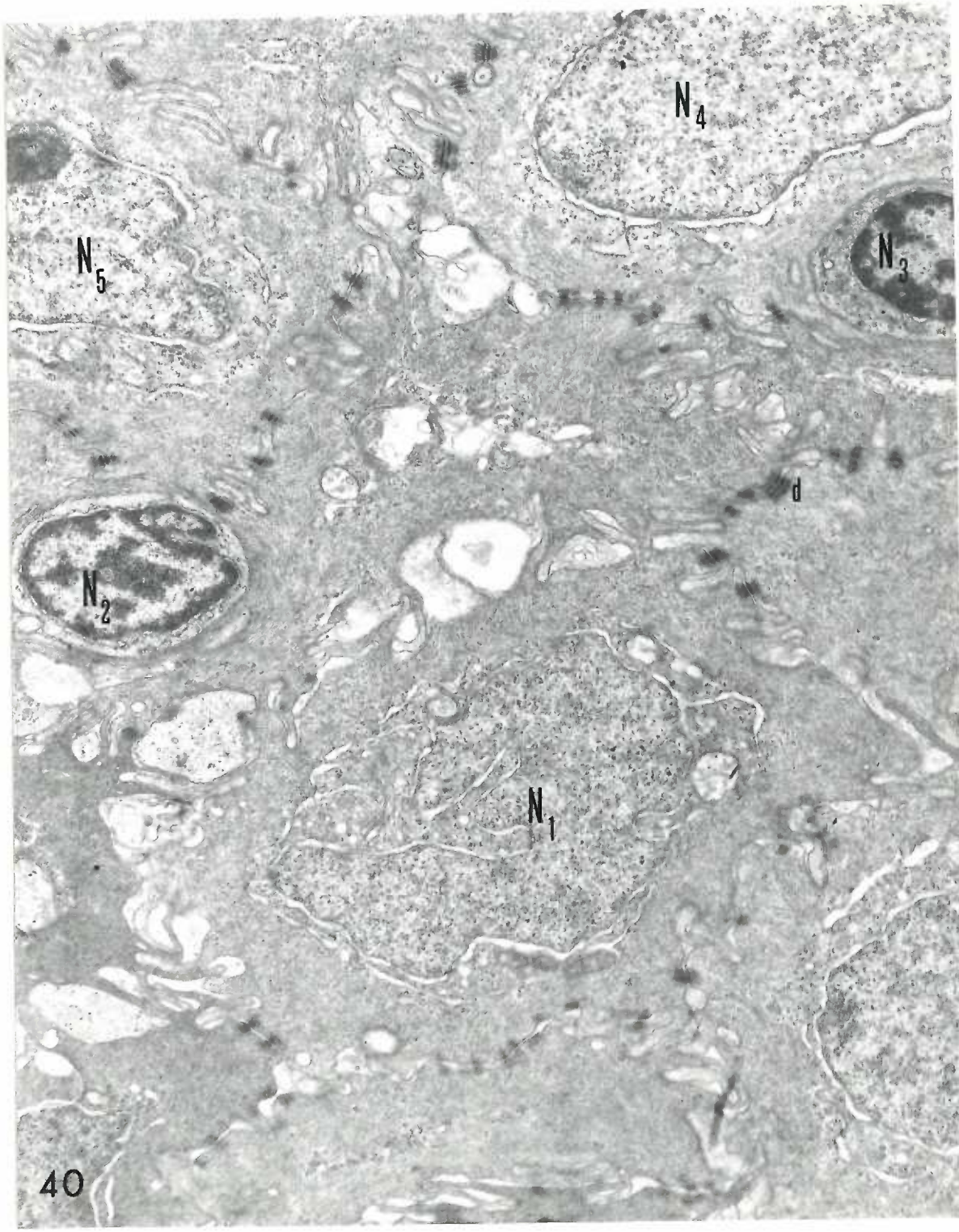

N4
N5
N3
d
N2
N1
40

Figure 41:

An electron micrograph of desmosomes exhibiting many cytoplasmic filaments (cf) connected to the desmosome plate and apparently passing through this structure (see arrow). At the bottom of this photomicrograph note the extensive interdigitation of adjacent cell membranes.

Lead citrate, uranyl acetate stain. 54,900X.

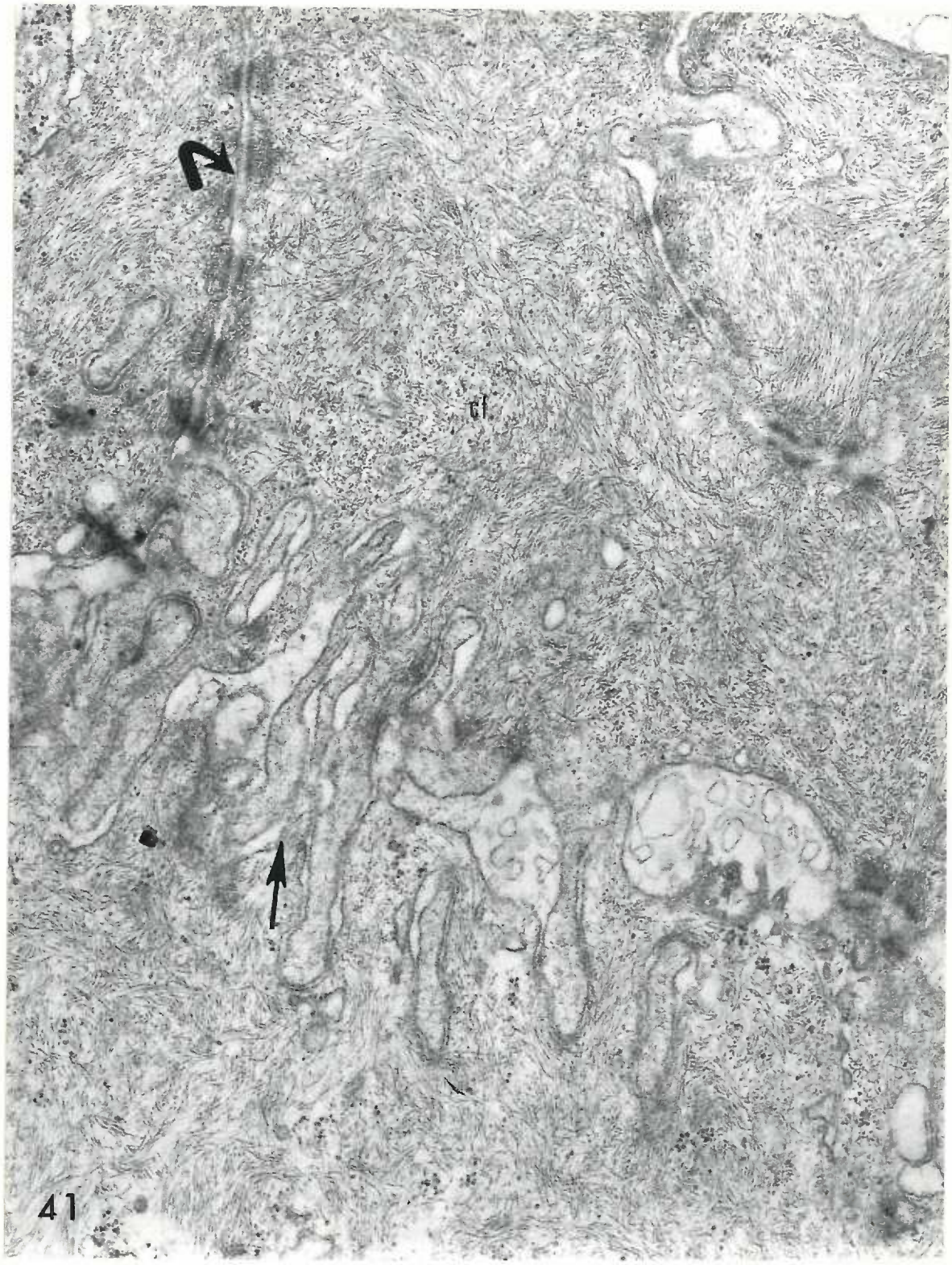

41

Figure 42:

An electron micrograph of age group I normal dermis. The arrow indicates the basement membrane. Eight to nine layers of dermal collagen (DC) are seen directly below the basement membrane. Underneath the dermal collagen is a fibroblast (FB) which contains mitochondria and a developed ergastoplasm. Erythrophores (EP), melanin granules, and iridocytes (IR) are also seen.

Lead citrate, uranyl acetate stain. 14,200X.

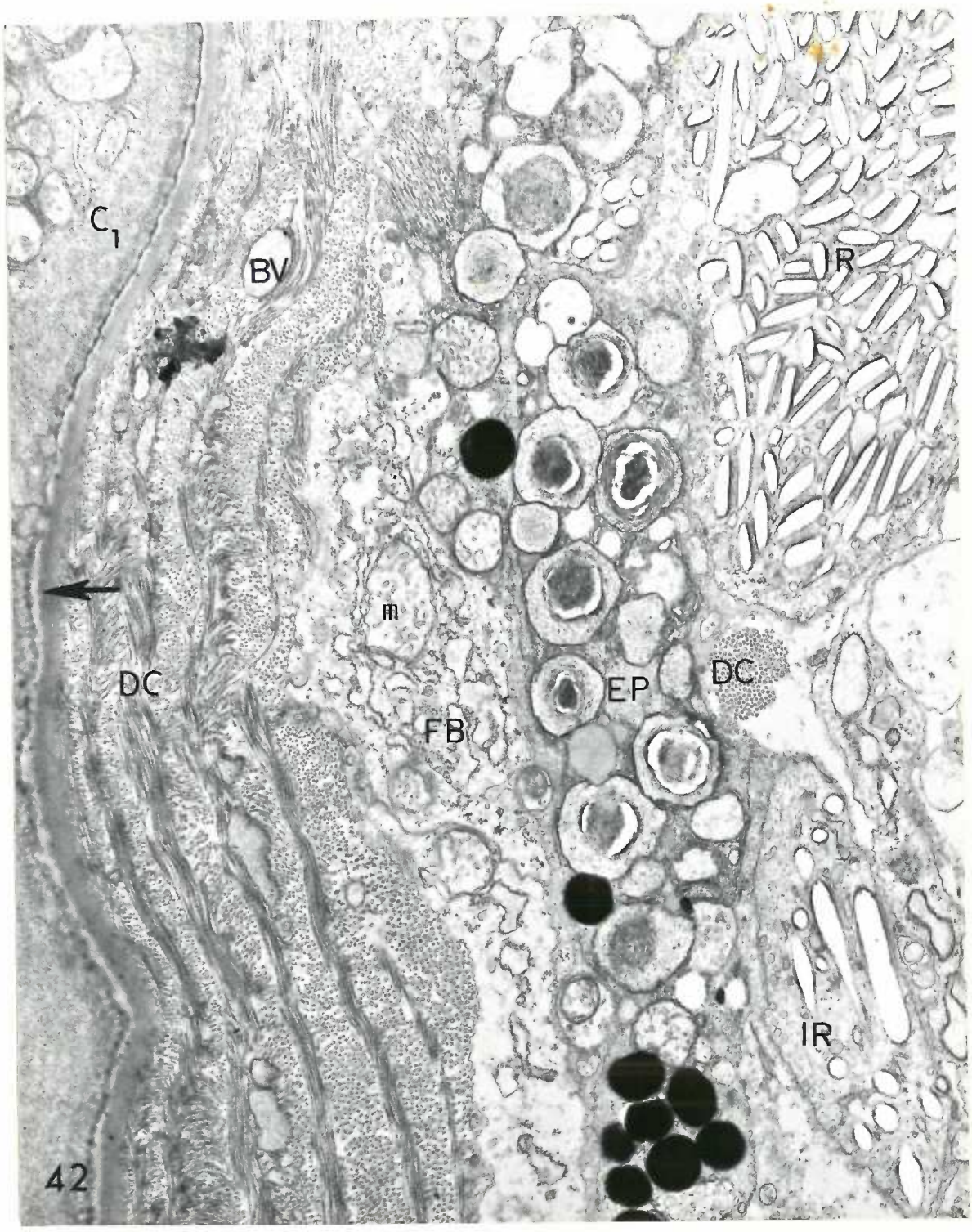

C1
BV
IR
DC
EP
DC
FB
IR
42

Figure 43:

An electron micrograph of squamous cells of the intermediate and superficial layers (top) of an angioepithelial nodule. Note the microvillous (mv) projections on the outer surface. Cells at both levels have normal numbers of intercellular connections. The nuclei of their cells vary in shape.

Lead citrate, uranyl acetate stain. 7,250X.

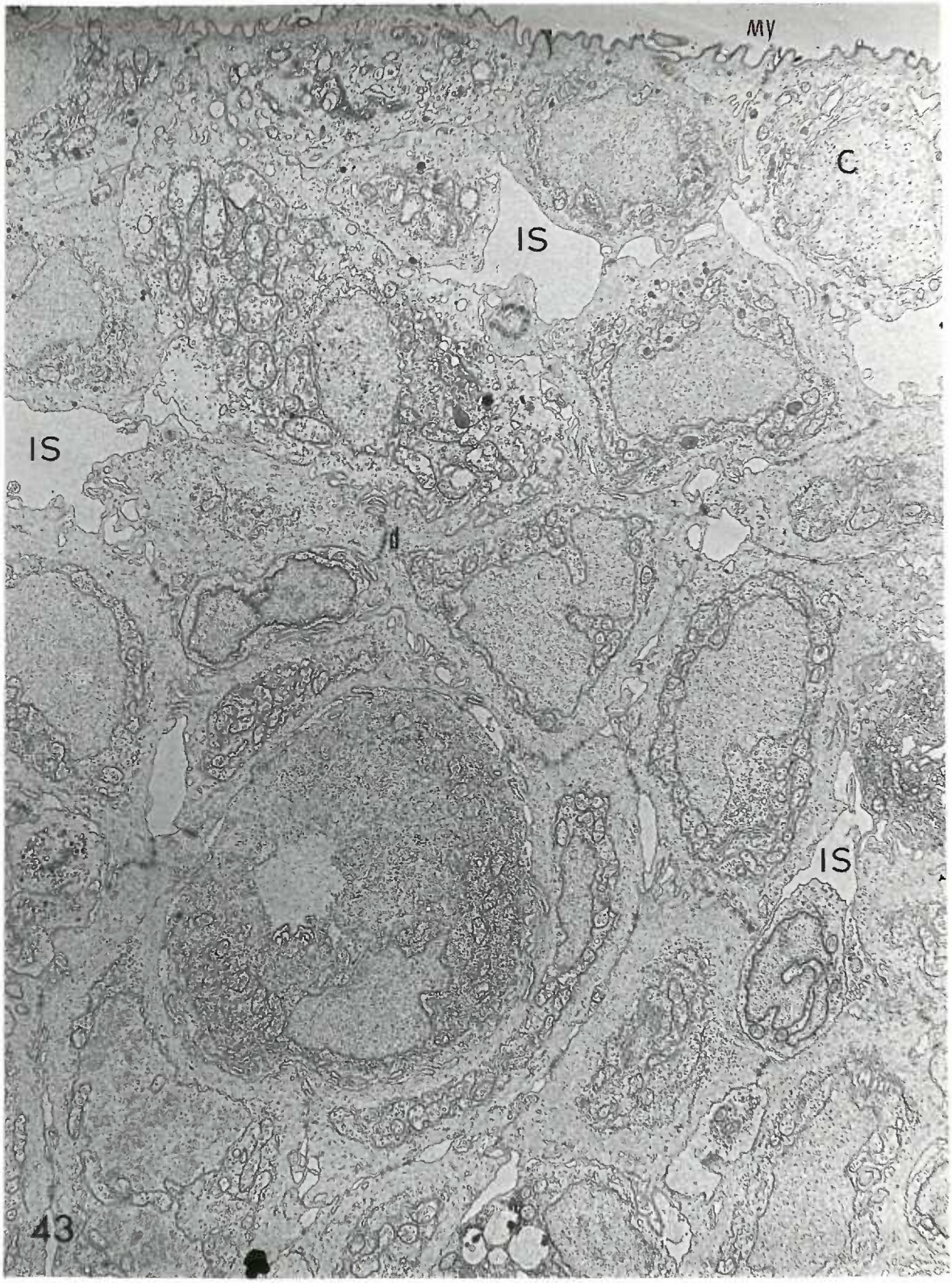

MY
C
IS
IS
IS
43

Figure 44:

An electron micrograph of basal and intermediate layers of an angioepithelial nodule. Note the poor development of the basement membrane (BM). Basal cells (C_1) are oriented perpendicular to the surface of the epidermis. The nuclei of these basal cells contain a nucleolus (see arrow). A mucous cell (M) is seen which contains a nucleus (MV), a developed ergastoplasm (er), Golgi apparatus, and many vesicles. Dermal collagen is not developed and appears to be organizing in the areas near fibroblast.

Lead citrate, uranyl acetate stain. 7,250X.

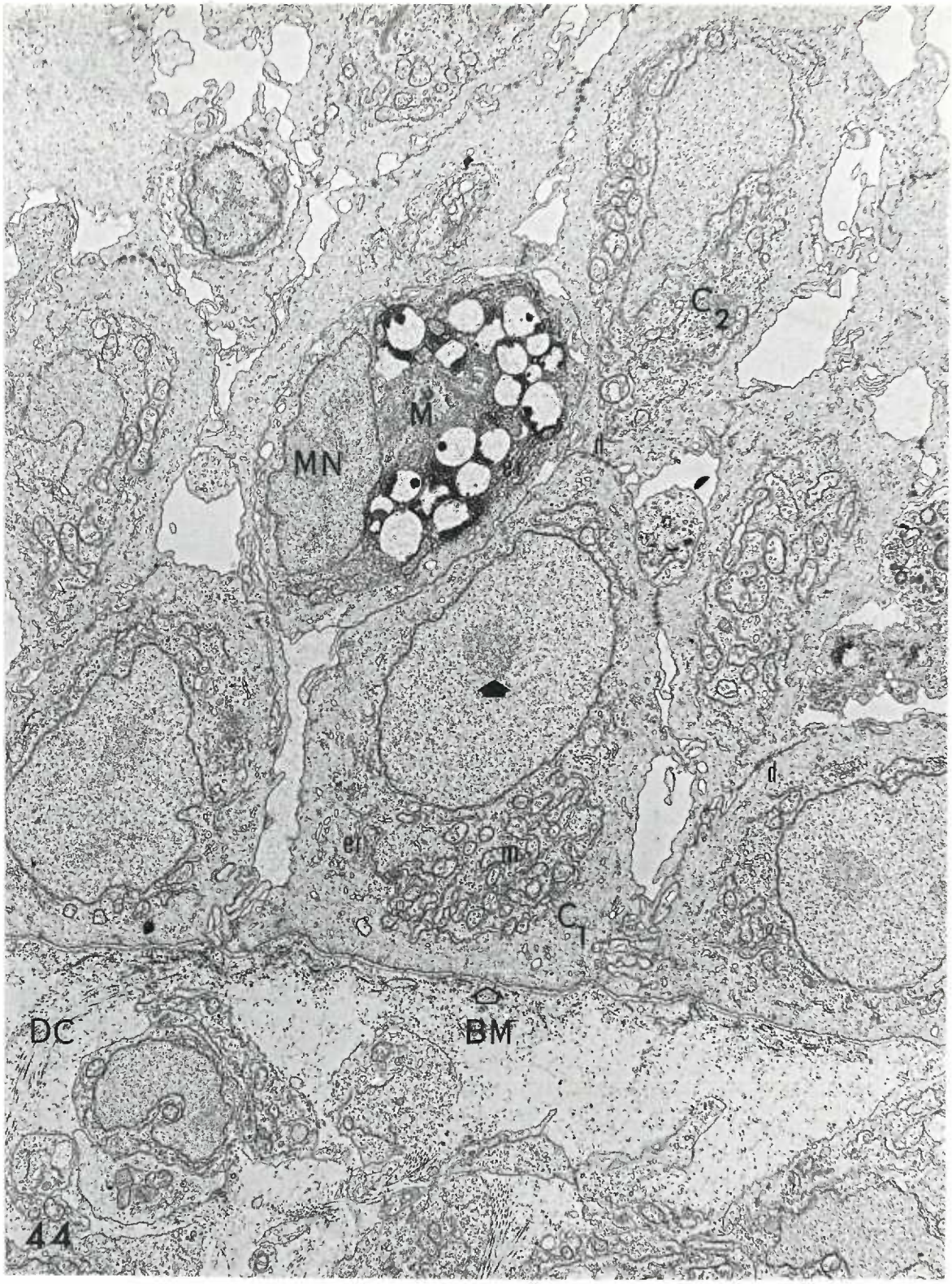

C2
M
MN
er
n
er
m
C1
DC
BM
44

Figure 45:

An electron micrograph of the dermis and epidermis of an angio-epithelial nodule. The arrow indicates the primitive basement membrane. The dermis (left) is undeveloped and shows a granular cell which contains dense inclusions in its cytoplasm and its nucleus contains a nucleolus (NC).

Lead citrate, uranyl acetate stain. 7,250X.

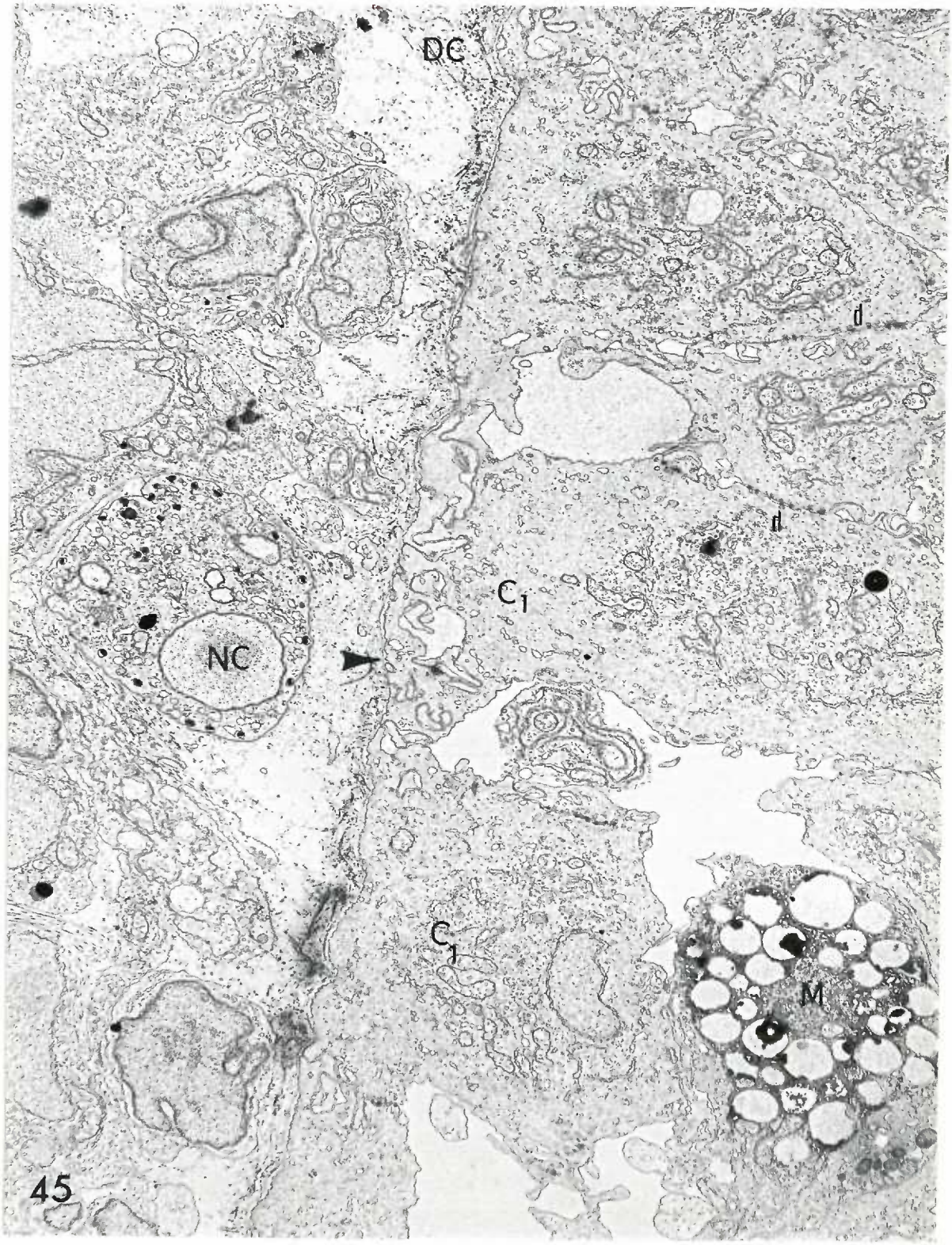

DC
d
d
NC
C1
C1
M
45

Figure 46:

An electron micrograph of the basement membrane (BM) of an angio-epithelial nodule. The dermal collagen (DC) is poorly developed and the faint line between the dermal-epidermal interface is the primitive basement membrane. The arrow indicates the interdigitation of adjacent cell membranes in the epidermis.

Lead citrate, uranyl acetate. 10,900X.

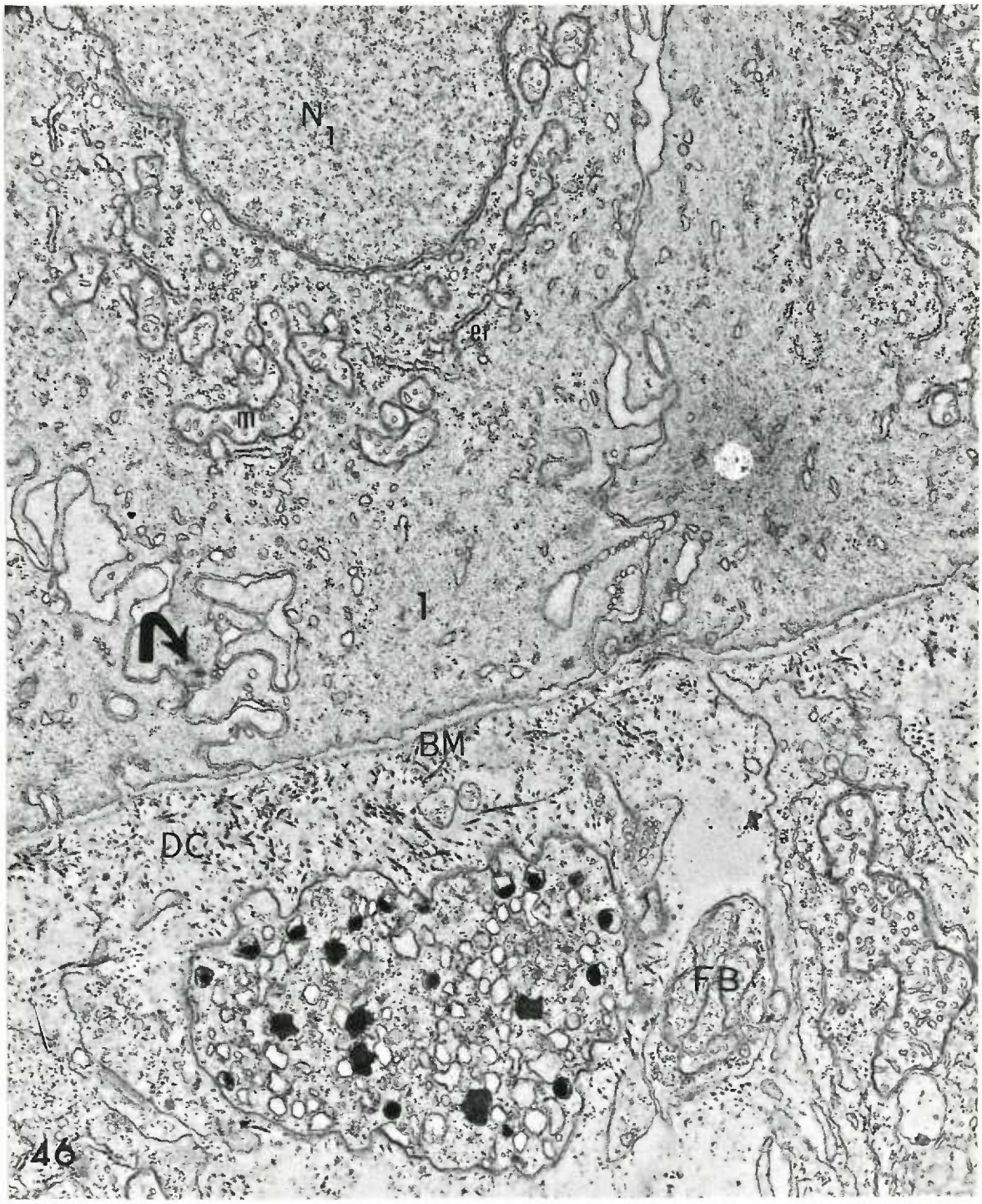
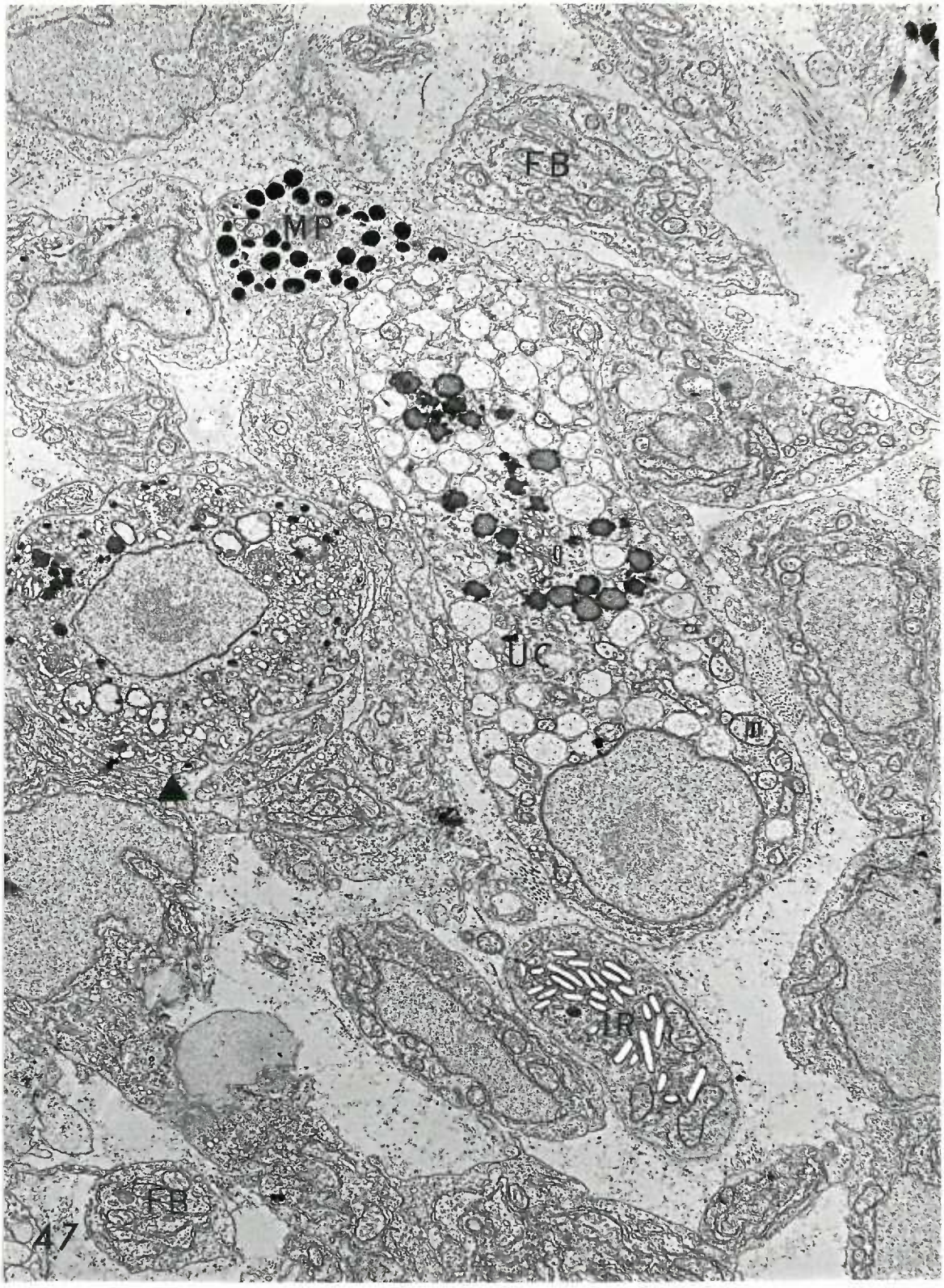

N
1
er
m
1
N
BM
DC
FB
46

Figure 47:

An electron micrograph of the deep dermis of an angioepithelial nodule. Note the large cell (UC) with many vacuoles (V) and dense granules. Also note the cell similar to the granular cell seen in Figure 45 (see arrow). An iridocyte (IR), melanophore (MP), and fibroblast are seen in the loosely connected structure.

Lead citrate, uranyl acetate stain. 7,250X.



47

Figure 48:

An electron micrograph of the epidermis of an angioepithelial nodule. Note the desmosomes (d) with their long cytoplasmic filaments (cf) and the interdigitation of adjacent cell membranes.

Lead citrate, uranyl acetate stain. 22,500X.

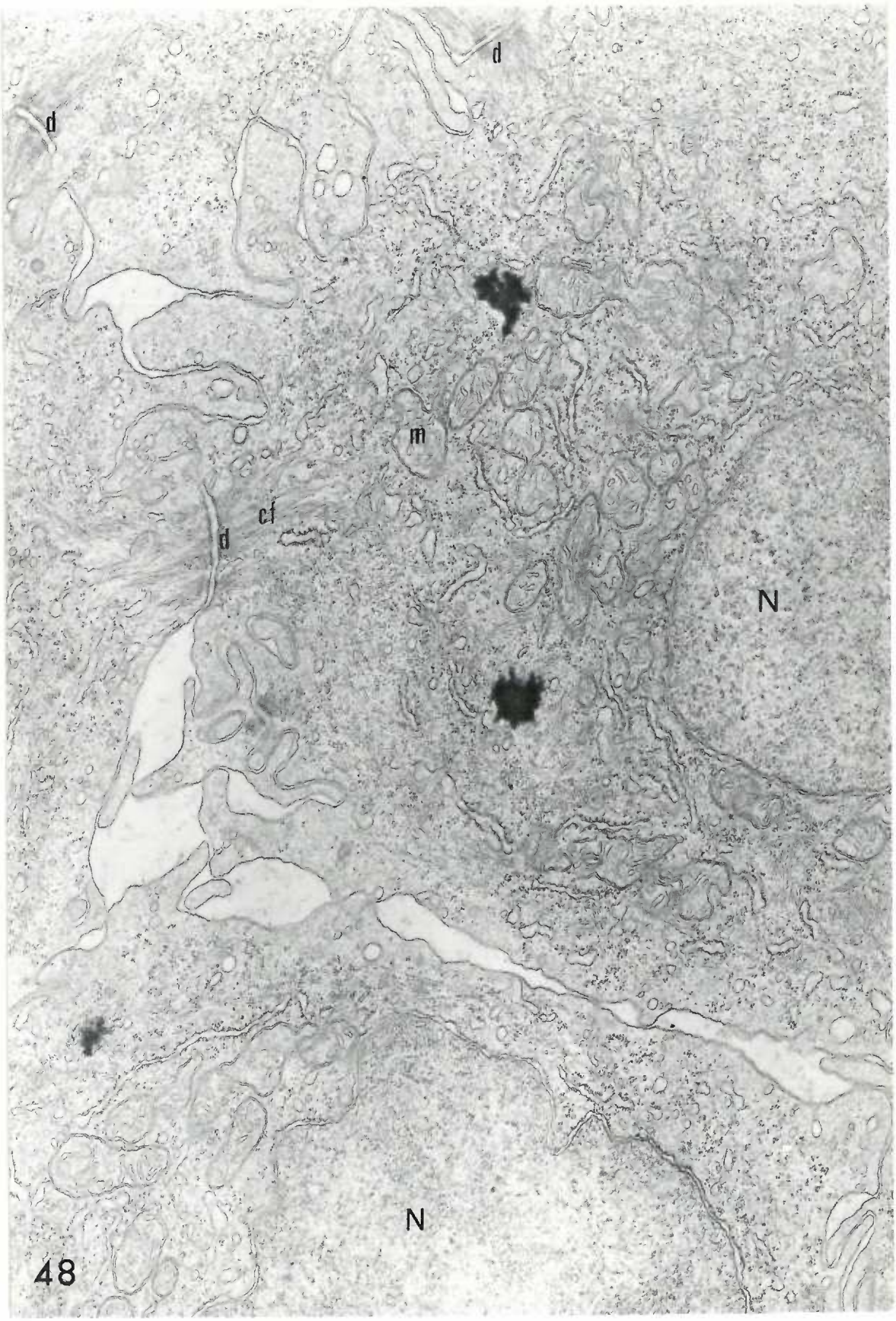
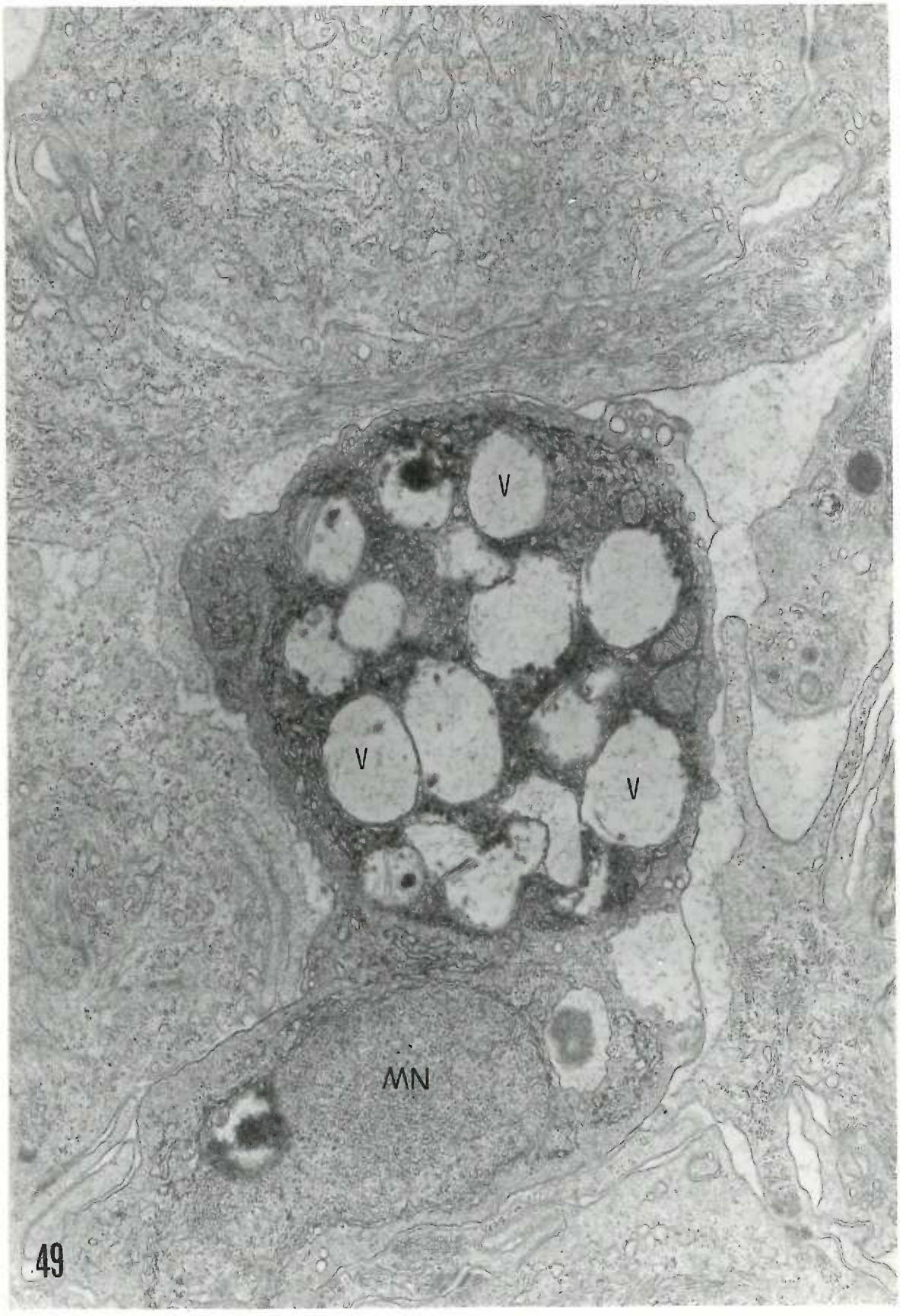

d
d
d
m
cf
d
N
N
48

Figure 49:

An electron micrograph of a mucous cell in the epidermis of an angioepithelial nodule. Note the mucous cell nucleus (MN), developed ergastoplasm, and many vesicles (v).

Lead citrate, uranyl acetate stain. 22,500X.



49

Figure 50:

An electron micrograph of the epidermis of an angioepithelial nodule. Note the pigment cell (Mc) contains various stages of melanin development (see arrow).

Lead citrate, uranyl acetate stain. 22,500X.

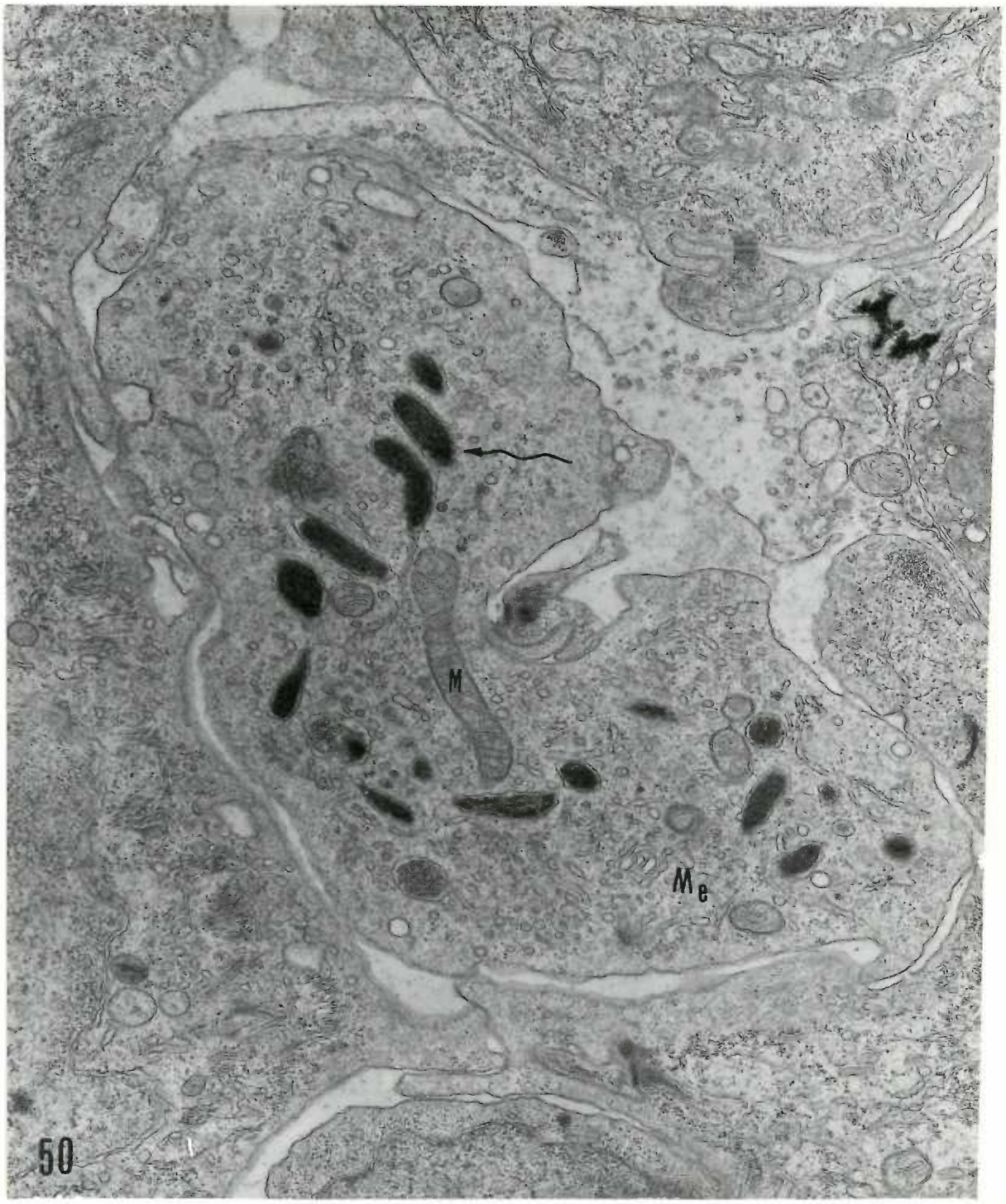

M
Me
50

Figure 51:

An electron micrograph of epidermal cells of an angioepithelial nodule. The arrow indicates a myelin-like figure surrounded by mitochondria (m) and ergastoplasm (er), and a well-developed Golgi apparatus (g).

Lead citrate, uranyl acetate stain. 22,500X.

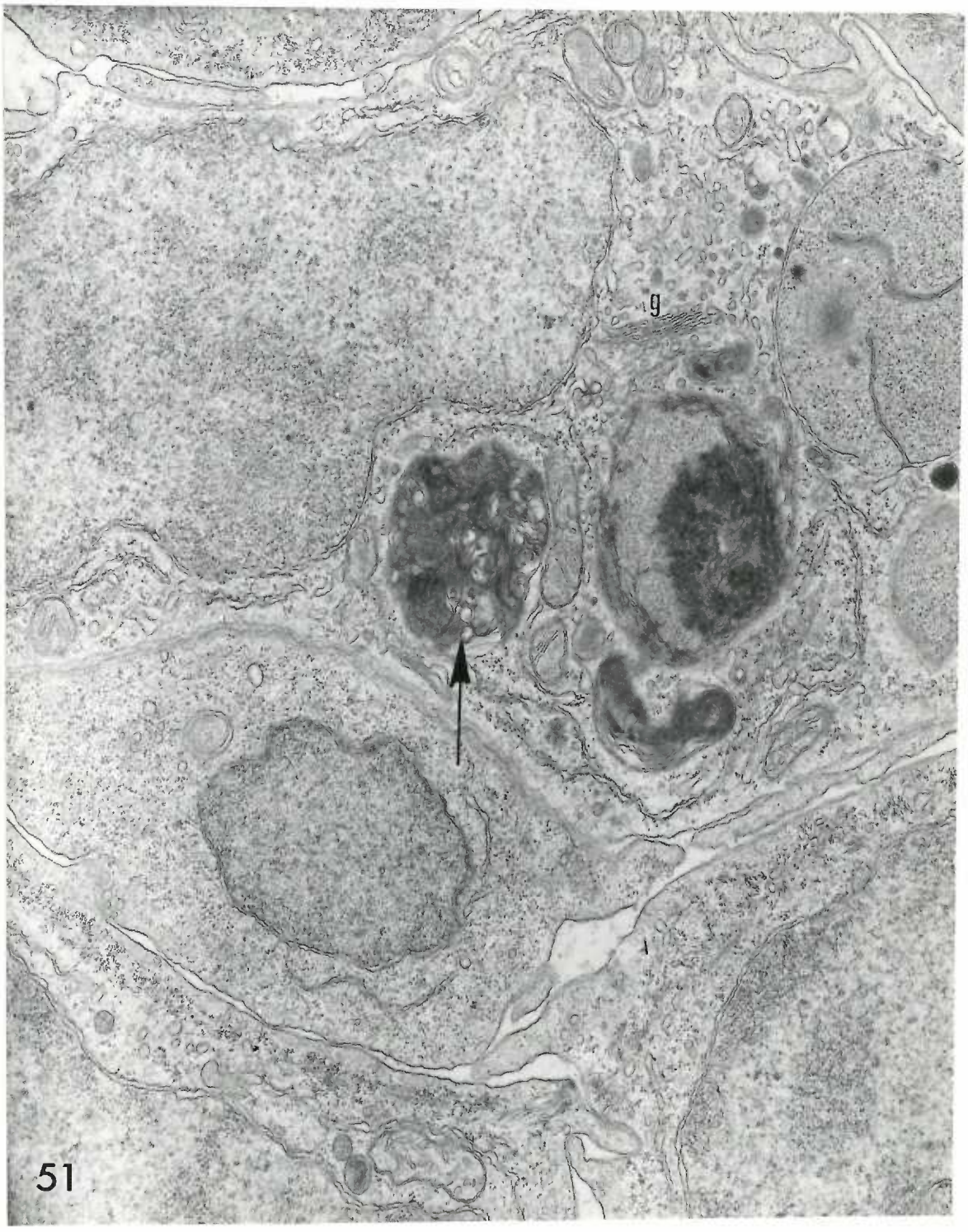
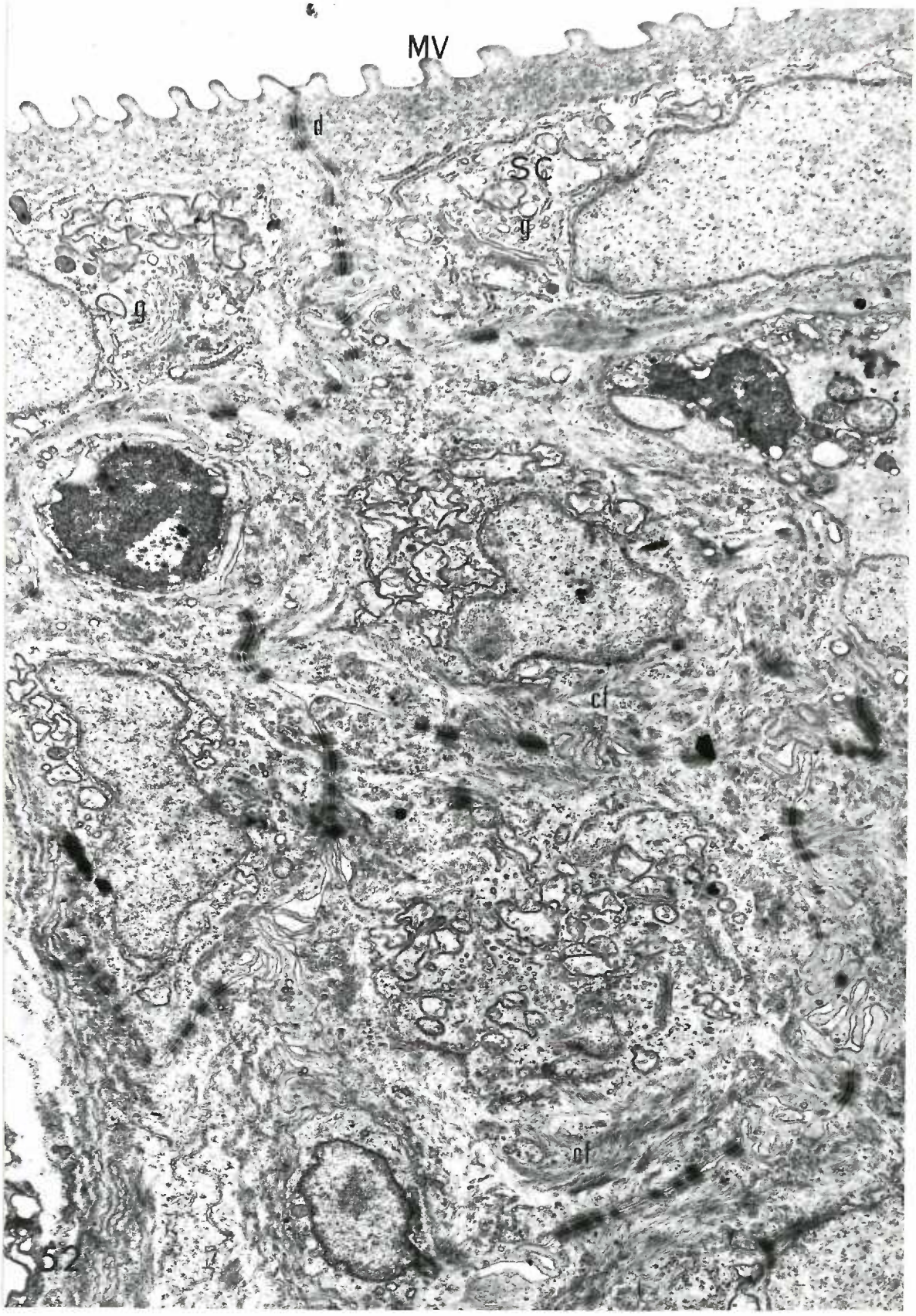

g
51

Figure 52:

An electron micrograph of the squamous cells of the intermediate (bottom) and superficial layers (top) of an epidermal papilloma. The outer surface is characterized by microvillous structures (mv). A dense layer of cytoplasm beneath the microvillous structures follows their contours. The superficial surface cells are slightly flattened and contain nuclei (N), ergastoplasm (er), and Golgi apparatus (g). The margins of the intermediate cells are indistinct and contain many desmosomes (d) and cytoplasmic filaments. Those cellular organelles mentioned in the superficial cells are also present in the intermediate cells.

Lead citrate, uranyl acetate stain. 9,900X.

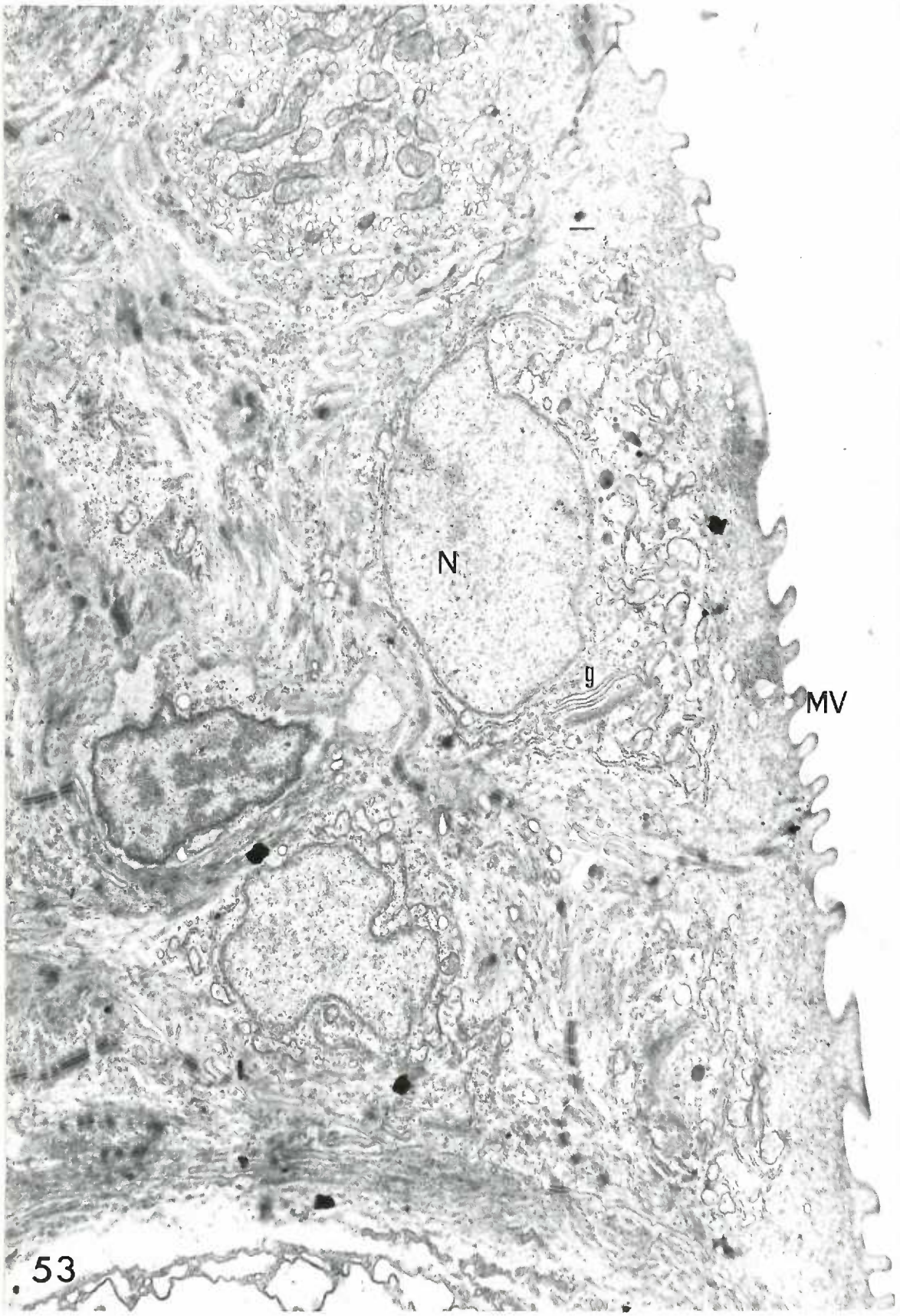


52

Figure 53:

An electron micrograph of the squamous cells of the intermediate (left) and the superficial layers (right) of an epidermal papilloma. The nucleus (N) of the superficial cell is irregular in shape and finely granular in consistency. The perinuclear cytoplasm contains the Golgi apparatus (g) and many ergastoplasmic sacs. A dense area lies just beneath the microvillous projections (mv) on the superficial surface.

Lead citrate, uranyl acetate stain. 11,550X.

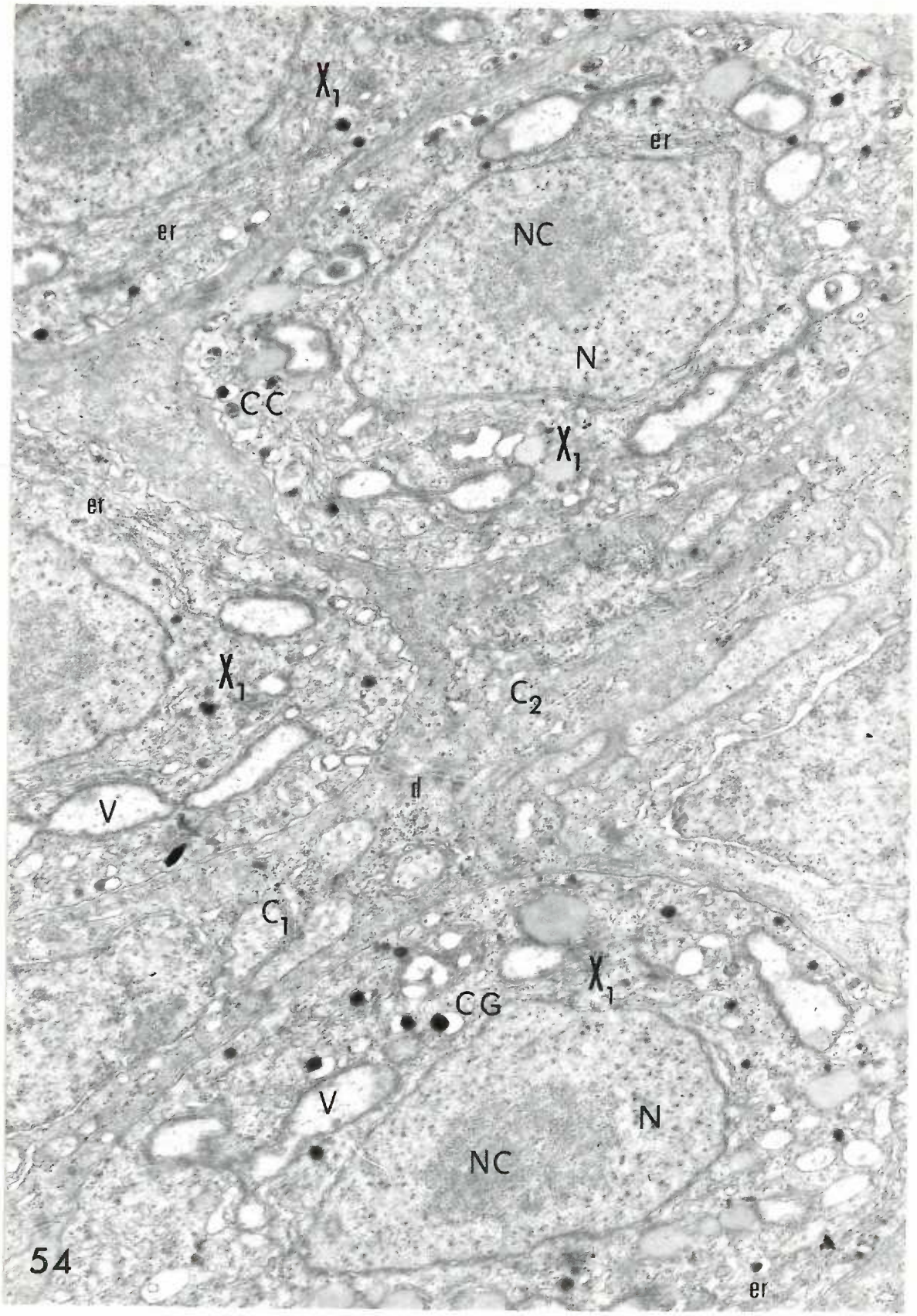


53

Figure 54:

An electron micrograph of several cells in papilloma tissue. These ovoid (X_1) cells contain a nucleus (N) which is slightly granular in texture. A dense nucleolus (NC) is seen within the nucleus. The double nuclear membrane is seen. The cytoplasm of these cells contains much ergastoplasm (er) and many vacuoles which may be degenerate mitochondria. Cytoplasmic granules (CG) are a prominent feature of these X_1 cells. The cytoplasmic membranes of these cells do not interconnect with adjacent cells. Note that the basal cell (C_1) and the intermediate cell (C_2) interconnect by desmosomes (d) and intermembrane interconnection. This cell is very similar to the granular cell seen in the dermis of Figures 45 and 57.

Lead citrate, uranyl acetate stain. 12,600X.



54

Figure 55:

An electron micrograph of X_1 cells seen in epidermal papilloma tissue. Note the long projecting processes (right) extending from the basal layers of the epidermis. The arrow indicates the cellular interconnections of two adjacent cells. No such interconnections are seen with the X_1 cells.

Lead citrate, uranyl acetate stain. 10,500X.

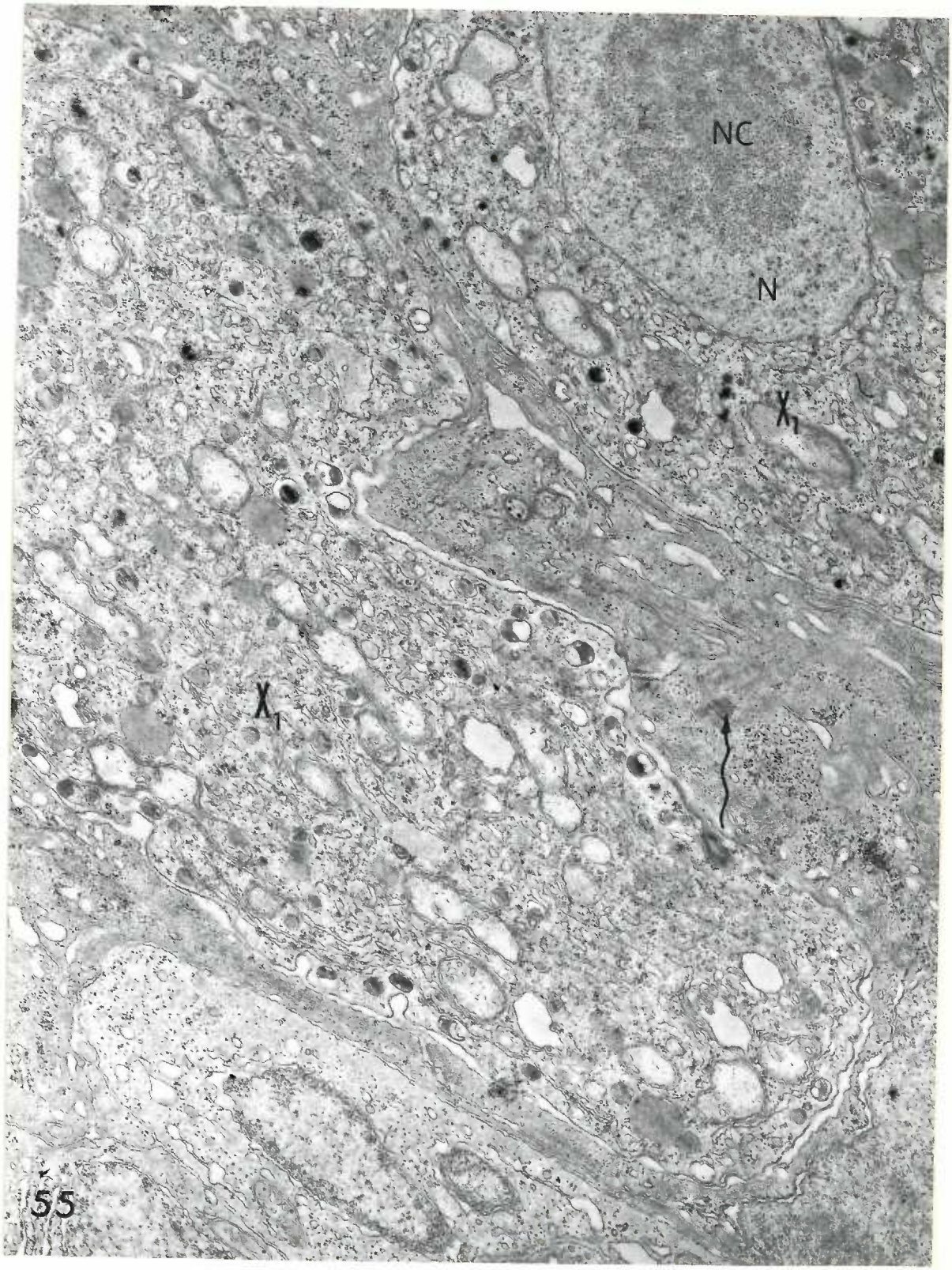

NC
N
X_1
X_1
55

Figure 56:

An electron micrograph of the epidermis and dermis of an epidermal papilloma. Note the marked interdigitation of adjacent membranes in the basal layer of the epidermis. The arrow indicates a whorled structure in the perinuclear position of an intermediate cell. The basement membrane (BM) is well formed at this time. The compact dermis (DC) is composed of 5 to 6 layers. Below these layers are seen cells with melanin granules and iridocytes (IR).

Lead citrate, uranyl acetate stain. 13,230X.

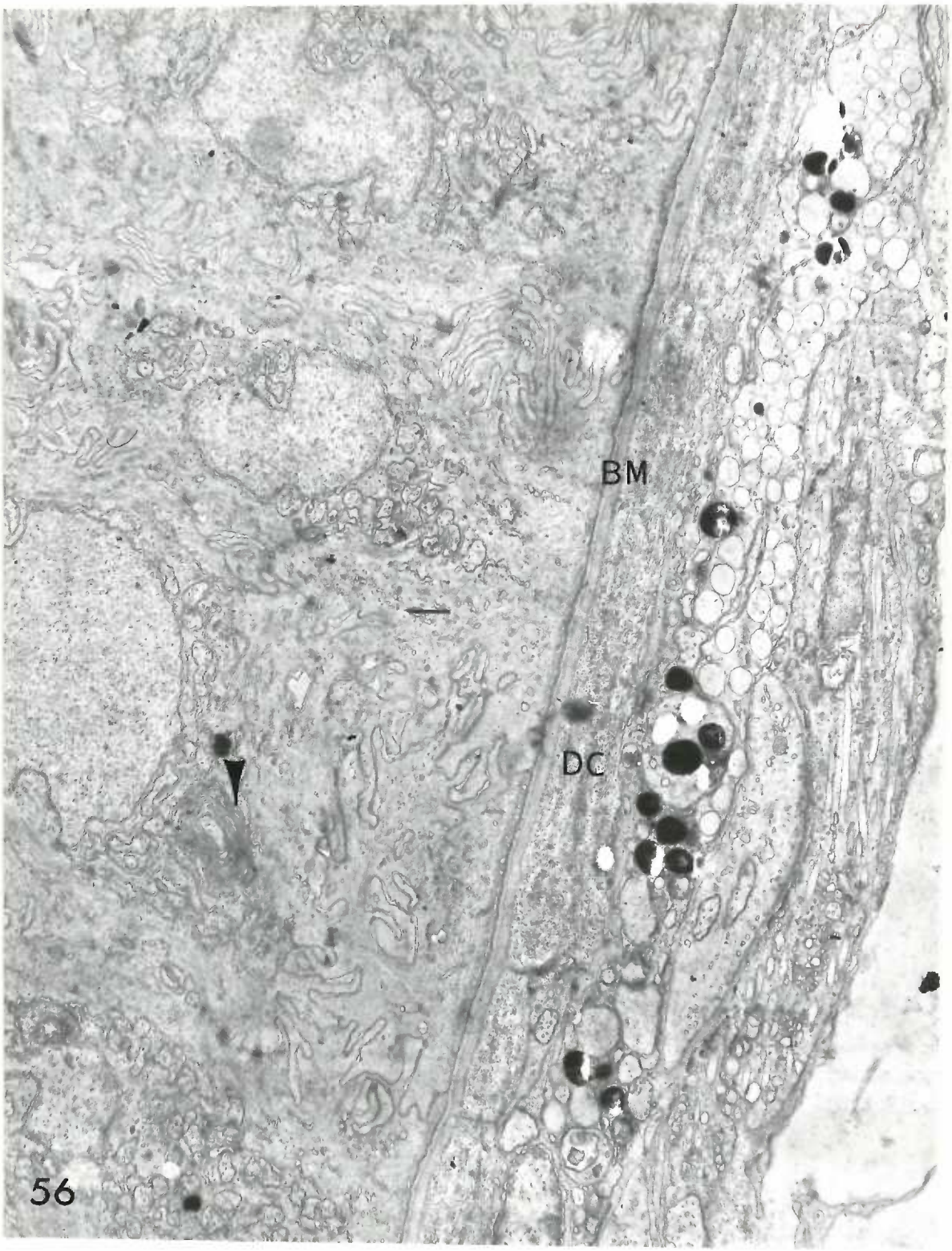

BM
DC
56

Figure 57:

An electron micrograph of dermis of an epidermal papilloma. The arrow indicates a cell in the dermis (right) that is similar to the X_1 cells seen in the epidermis of papilloma tissue (Figs. 54-55). Fibroblast (FB) and melanophores (me) are also seen in the dermis. The compact dermal collagen (DC) is composed of 5 to 6 layers and is separated from the epidermis (top left) by a developed basement membrane. Note the X_1 cells in the upper left hand corner.

Lead citrate, uranyl acetate stain. 10,500X.

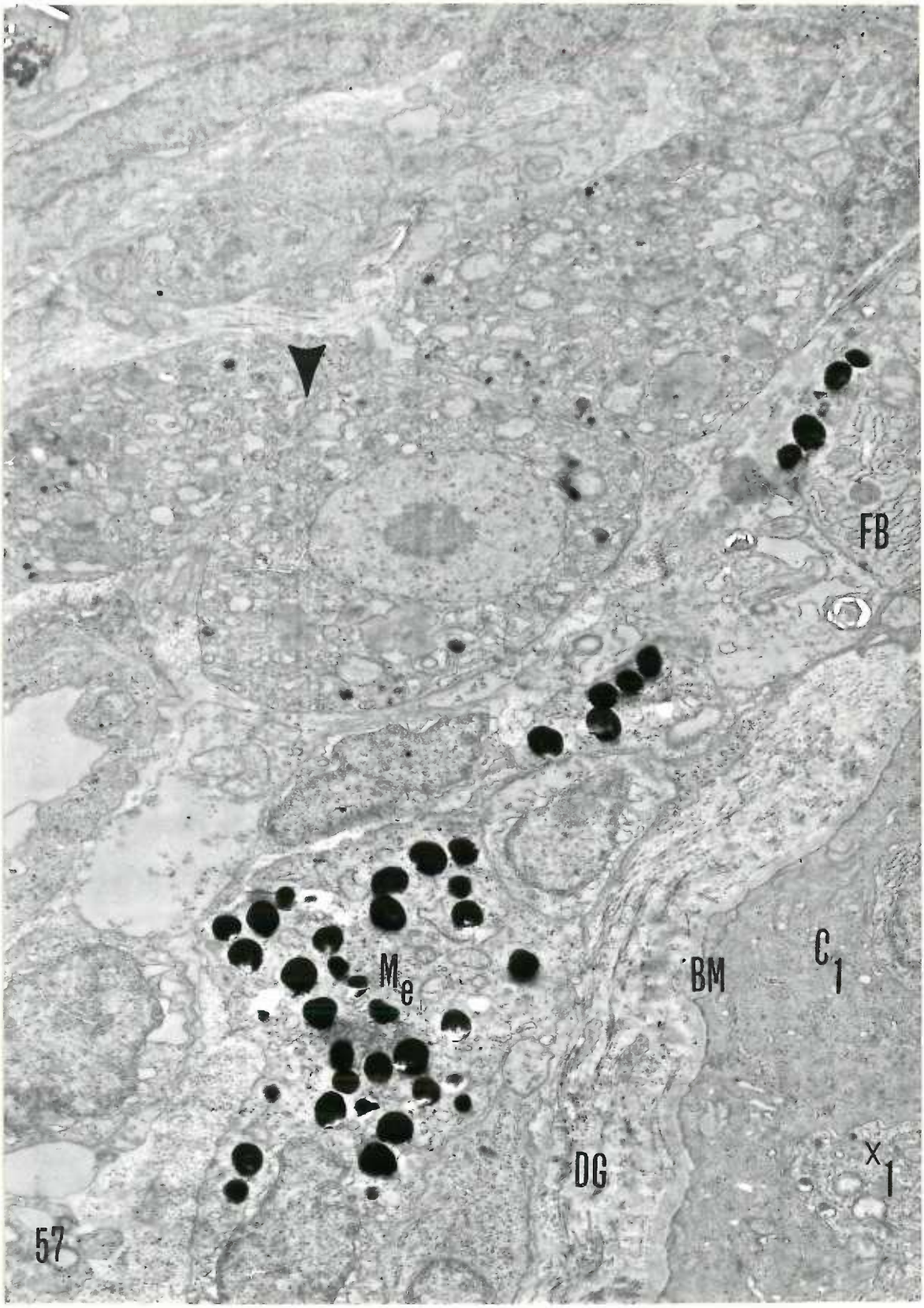

FB
M_e
BM
C_1
DG
X_1
57

Figure 58:

An electron micrograph of virus particles in a crystalline array, found in an angioepithelial polyp.

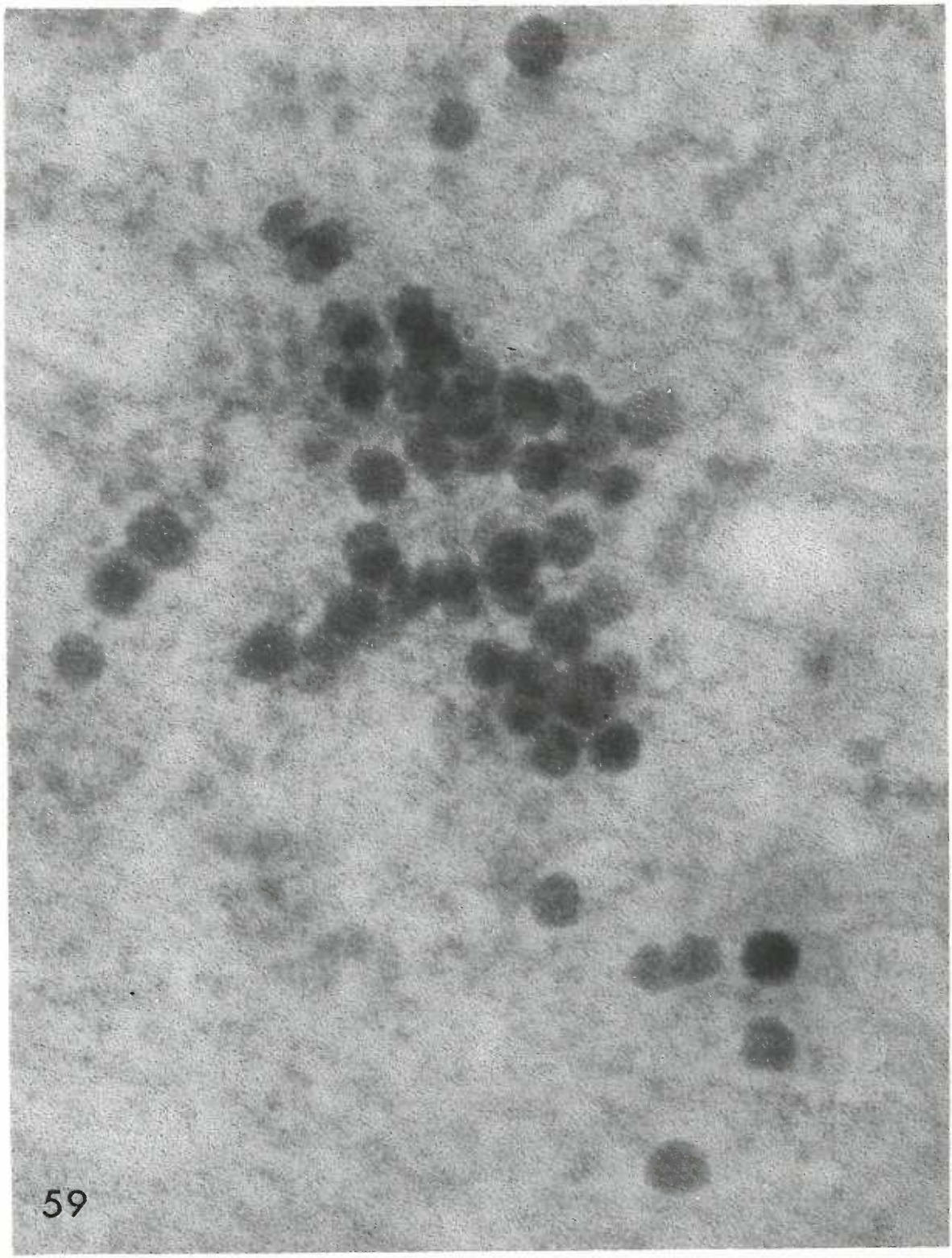
Lead citrate, uranyl acetate stain. 47,700X.

58

Figure 59:

An electron micrograph of virus particles found in an angio-epithelial polyp.

Lead citrate, uranyl acetate stain. 335,160X.



59