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LIBBY ROSENBLOOM FRIEDMAN

The American Museum of Natural History
and New York University
Presently at the Department of Pathology,
Mount Sinai Hospital, Cleveland, Ohio

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INTRODUCTION

THE COMPREHENSIVE BIBLIOGRAPHY of the research in tissue culture of Murray and Kopech (1953) impresses one with the relatively few studies on cold-blooded animals, despite the fact that pioneer work in tissue culture was done with frogs. Especially scarce are investigations that utilize teleost tissue, particularly of the adult. Since 1953, there have been several *in-vitro* studies on normal adult teleost tissues (Wolf and Dunbar, 1957; Wolf, Quimby, Pyle, and Dexter, 1960; Hu and Chavin, 1960; Kim, Tchen, and Hu, 1961) as well as malignant teleost tissue (Greenberg, Kopac, and Gordon, 1956; Greenberg and Kopac, 1961).

In the laboratories of the Department of Ichthyology at the American Museum of Natural History, extensive studies were initiated on all aspects of the biology of *Astyanax mexicanus* and the derived Mexican cave fishes of the genus *Anoptichthys*. In the course of this work, two spontaneous lymphosarcomas, reputedly of thymic origin, appeared in laboratory-reared *Astyanax*. These tumors focused our attention on normal and abnormal aspects of the thymus and led to several studies on the problem of growth in general (Rasquin, 1951; Rasquin and Atz, 1952; Hafter, 1952; Rasquin and Rosenbloom, 1954). The work reported here represents the final paper in this series of studies on the lymphoid and endocrine tissues of these fishes.

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MATERIALS AND METHODS

NORMAL THYMUS

THE THYMUS GLANDS of approximately 200 specimens of *Astyanax mexicanus* (Fillipi) were cultured for this study. The standard lengths of the fish used ranged from 16 mm. to 46 mm., with a mean value of 28.5 ± 6.08. Owing to the fact that the thymus in this species starts involuting at an early age (Hafter, 1952), fish selected for use were in the range of two to nine months. Early experiments indicated that the thymuses most satisfactory for culturing were those of two- to three-month-old fish, with a standard length of approximately 25 mm. At this time, the gland is large, resilient, and readily accessible for extirpation. For each culturing period, fish of approximately the same age and size were selected.

Standard sterility procedures were observed. In addition, because of the immediate subdermal location of the thymus gland, which is not easily separated from its skin covering, extreme precautionary measures for asepsis of the tissue were necessary. The fish was first anesthetized in 1 per cent urethane, then placed in a 0.01 per cent solution of sodium-merthiolate for five minutes. The gland was removed under the dissecting microscope with the aid of watchmaker forceps and was placed in sterile Holtfreter's solution containing penicillin G (100 units/cc.) and streptomycin (0.01 mg./cc.) for at least 15 minutes before being cultured. The antibiotics were also included directly in the culture media in the same concentrations. There was consequently no bacterial growth in any of the cultures. It was later found that, with the use of ultraviolet irradiation to sterilize the culture room, the streptomycin could be eliminated; the penicillin concentration, however, was increased to 1000 units per cubic centimeter.

The medium consisted of Holtfreter's solution, chick embryo extract, chicken plasma, and carp serum. In some experiments, human ascites fluid, obtained from ovarian malignancies, was substituted for the serum.

The Maximow double cover-slip method of culturing was employed, and a total of 505 thymus cultures were made. Pieces of tissue approximately 1 cubic mm. in size were used to start the cultures.

For most of the routine morphological studies, a solid medium was used. The fragment was embedded in two drops of a mixture of plasma (one part), embryo extract (one part), and carp serum (two parts). For the histochemical studies, in which a staining of the clot often obscured the reaction in the cells, a liquid culture medium was found to be more useful. To afford some anchorage for the cells and to delimit the area over which they might proliferate, the cover slips were first plasma-coated, with a small amount of the following mixture: plasma (one part), embryo extract (one part), and Holtfreter's solution (three parts). After the clot had formed, the fragment and fluid nutrient medium were added. The fluid medium consisted of embryo extract (one part), Holtfreter's solution (two parts), and carp serum (two parts).

Trypan blue, at a concentration of 1:100,000, as suggested by Muhlethaler (1952), was included in the culture medium of several cultures in order to help establish the phagocytic nature of some of the cells.

Cultures made during the cool months were stored at room temperatures, while those made during the warmer months were kept on a water table where an average temperature of 25° C. was maintained.

Observations of the cultures were made daily to study the extent and nature of growth. Representative cultures were fixed in Bouin's fluid one, two, three, four, five, seven, and 14 days after explantation and routinely stained with Harris' haematoxylin and eosin for further study. To help clarify the structure of the thymocytes, additional cultures were fixed and stained with Mayer's haemalum, Wright's stain, and the Dominici stain. Measurements of thymocyte nuclear diameters were made with the aid of a stage micrometer on 100 cells in representative haematoxylin and eosin-stained cultures, and the mean was determined.

A series of histochemical determinations was made in order to obtain additional information about the cells in general, and in particular to help elucidate the nature of epithe-

lial inclusions. This series was divided into three groups: nucleoproteins, carbohydrates, and lipids. In the first group, the presence of nucleic acids was demonstrated with methyl green-pyronin, with and without ribonuclease digestion (Taft, 1951; Pearse, 1960).

In the second group, a variety of techniques was used to distinguish between glycogen and mucopolysaccharides. These included Best's carmine (Pearse, 1960), the Bauer-Feulgen method (Bensley, 1939; Deane, Nesbett, and Hastings, 1946), and the McManus' Periodic Acid-Schiff technique (Purves and Griesbach, 1951). Control preparations were made after exposure to saliva. In addition, Mayer's mucicarmine (Cowdry, 1952) and Alcian blue (Steedman, 1950) were also used to demonstrate further the presence of mucin.

Total lipids were demonstrated with Sudan Black B (Lillie, 1948), while Sudan IV (Lillie, 1944) was used to demonstrate neutral fats. Baker's Acid Hematein method (Baker, 1946) was employed for the staining of phospholipids; the controls were similarly treated after previous extraction with pyridine.

To determine whether the adrenal hormones affect lymphoid tissue directly at the cellular level or whether these effects are dependent upon intact glandular interrelationships, cultures of normal thymus tissue were treated *in vitro* with cortisone acetate (Merck and Co.) and whole mammalian adrenal cortical extract (Wilson Laboratories). In order to study the continuous effects of the hormones, they were incorporated directly in the culture medium. Cortisone suspensions were made with Holtfreter's solution, and the concentrations per culture were 1.6 μg., 0.08 μg., 0.008 μg., and 0.0008 μg. An equivalent amount of Holtfreter's solution, as used with cortisone, was included in the medium of the controls. Representative cultures were fixed in Bouin's fixative five, 10, 24, 48, 72, and 120 hours after explantation, and stained with Harris' haematoxylin and eosin.

Similar cultures were set up that incorporated whole adrenocortical extract in the medium in concentrations of 20.0 per cent, 10.0 per cent, 2.0 per cent, and 0.2 per cent per culture. The saline content of the concentrated extract (derived from 75 mg. of fresh adrenal tissue suspended in 0.85 per cent sodium chloride) was adjusted to 0.6 per cent to be physiologically isotonic for fresh-water fish. Controls were made with 0.6 per cent saline. Representative cultures were fixed and stained 24, 48, 72, and 168 hours after explantation.

To determine the recuperative capacity of lymphoid tissue subjected to the temporary effects of cortisone, a second series of cultures was made in which the intact thymuses were first incubated in 0.5 cc. of Holtfreter's solution, containing 5.0 μg. cortisone, for one, one and a half, or three hours. Controls were incubated in a similar quantity of Holtfreter's solution for the same periods of time. Cultures were then made, using the routine medium, and were sacrificed approximately at one, five, or 24 hours after explantation.

Owing to the erratic nature of thymocyte migration from the thymus grown in tissue culture, a comparison of the percentages of normal cells and those with pycnotic nuclei could not be considered a valid criterion of the effect of the adrenocortical hormone. Sometimes one region of migration was composed exclusively of healthy thymocytes, while immediately adjoining was a zone of disintegrating cells. Since tissue sections of the thymus gland do not reveal such erratic distribution of normal and pycnotic cells, whole thymuses were incubated for 24 hours in 1-cc. quantities of Holtfreter's solution containing, respectively, 0.1 μg., 1.0 μg., 10.0 μg., and 200.0 μg. of cortisone. Holtfreter's solution without the hormone served as a control. The glands were fixed in Bouin's fixative, and sections were cut at 7 μ and stained with Harris' haematoxylin and eosin. Approximately 2000 cells each were counted from the peripheral and central regions of the thymuses, and the percentage of cells with normal or pycnotic nuclei was determined.

LYMPHOSARCOMA

During the course of work at our laboratory at the American Museum of Natural History, two spontaneous lymphosarcomas, reputedly of thymic origin, were noted. The first (Tumor A), occurred in a six-year-old male hybrid, a cross between *Astyanax mexicanus* (Filippi) and a blind cave derivative, *Anoptichthys jordani* (Hubbs and Innes). The second tumor (Tumor B) appeared several months later in a 15-month-old male *Astyanax*. In

both instances biopsies were taken from the tumor mass protruding from the left branchial cavity and were used for routine histological study as well as for tissue cultures. The first lymphosarcomatous fish was subsequently treated with ACTH in an attempt to inhibit the tumor growth (Rasquin and Hafter, 1951). Transplants were made from additional biopsy material of the second lymphosarcoma into both light- and dark-reared *Astyanax*, in connection with a study on endocrine imbalance and tissue hyperplasia (Rasquin and Rosenbloom, 1954). It was hoped that the transplantations would furnish an additional supply of tumorous tissue for further investigations *in vitro*. None of the transplanted tumor fragments, however, became established in the hosts.

The biopsy material was cultured, with the use of the standard double cover-slip method of Maximow. The basic medium consisted of chicken plasma, chick embryo extract (containing penicillin and streptomycin), and carp serum in the proportion of 1:2:1. Twenty-eight cultures were made from the first tumor and 24 from the second tumor. Representative cultures were subjected to cortisone,[1] compound A, whole adrenocortical extract, and adrenocorticotropic hormone in order to determine whether or not the lymphocytes were affected directly at the cellular level. The dosages used are given in table 1. The substances to be tested were either incorporated in the medium, so that the cultures were under their continuous influence from the time of explantation, or they were contained in a wash so that the cultures were subjected only temporarily to their effects. In the latter group, 48-hour cultures were opened, subjected to the wash, and then were resealed and allowed to continue to grow. All cultures were sacrificed on the second, third, or fourth day. They were fixed in 10 per cent formalin and were stained with Harris' haematoxylin and eosin.

TABLE 1

EXPERIMENTAL PROCEDURES FOR THE TREATMENT OF CULTURES OF TUMOR BIOPSY MATERIAL

Substance Used	Tumor No.	Actual Concentration In Wash	In Medium
Experimentals			
Cortisone	1	(3)[a] 5.0 μg. in 10 cc. Holtfreter's solution	—
	2	(2) 5.0 μg. in 10 cc. Holtfreter's solution	(3) 0.02 μg./culture
Compound A	1	(3) 5.0 μg. in 10 cc. Holtfreter's solution	(1) 0.1 μg./culture
	2	(2) 5.0 μg. in 10 cc. Holtfreter's solution	(2) 0.02 μg./culture
Whole ACE	1	(2) 1:1000 dilution of stock	(2) About 1:2000/culture
	2	(2) 1:1000 dilution of stock	(2) About 1:9000/culture
ACTH	1	(2) 20 μg. in 10 cc. Holtfreter's solution	(1) 0.04 μg./culture
		(2) 100 μg. in 10 cc. Holtfreter's solution	(3) 0.008 μg./culture
		(2) 200 μg. in 10 cc. Holtfreter's solution	—
Controls			
Untreated	(4)1		
	(2)2		
Holtfreter's solution	1	(3)	
	2	(1)	
Distilled water	2		(3)

[a] Figures in parentheses refer to the number of cultures made.

[1] The cortisone and compound A were generously supplied by Dr. M. J. Kopac of New York University. The whole adrenocortical extract was donated by Wilson and Company, the ACTH, by Armour and Company.

OBSERVATIONS

NORMAL THYMUS

THE NORMAL THYMUS of *Astyanax* has been described by Hafter (1952) as a "paired, opaque, white, bean-shaped structure" situated immediately under the operculum. It lies in the superior corner of the branchial cavity in the angle formed by the operculum and the dorsal body musculature, and is attached to the branchial epithelium (pl. 39, fig. 1).

The thymus is surrounded by a connective tissue capsule and it is composed histologically of two major elements: a parenchyma made up primarily of lymphoid cells, and an epithelial reticulum. Owing to the extensive vascularization of the gland (pl. 39, fig. 2), occasional free red blood cells are also present. In addition, eosinophilic wandering cells, macrophages, and melanophores have been observed. These cell types are also recognizable *in vitro*. The morphological description that follows is based on living as well as fixed and stained cultures.

GROWTH PATTERNS AND CELL TYPES IN TISSUE CULTURE

THYMOCYTES AND THYMOBLASTS: The first cells to migrate within two hours after cultures of the thymus were made were the small thymocytes. The free cells seen throughout the field immediately after explantation were not the result of migration, but rather of mechanical manipulation of the fragment at the time the cultures were made.

Several types of migrations were observed, the most common of which is referred to as "halo-like" (Pappenheimer, 1913; Pfeiffer, 1935). Eventually, as the migration became more extensive, the halo effect (pl. 39, fig. 3), produced by the rapid concentration of cells in a small area, disappeared, and the thymocytes became more evenly dispersed.

Another frequent occurrence was that seen in figure 4 of plate 39 in which a smaller number of cells wandered out. Here the advancing edge of cells was quite regular, so that the over-all appearance was of a thin membrane. Actually no structural barrier was noted, and the entire migrating zone was occupied exclusively, but sparsely, by thymocytes. On several occasions, no additional migration or cellular proliferation occurred.

Occasionally, capillary-like formations were seen, similar to those described by Hueper and Russell (1932) for chicken leucocyte cultures. Figure 5 of plate 39 shows a three-day culture in which such a configuration occurred. These "tubes" began as projections or buds from the explant, which were filled with thymocytes. Although the tubes appeared to be clearly demarcated from the surrounding plasma by a hyaline-like membrane, no nuclei were ever detected.

In general, the thymocytes were of two types: the small, round, refractile cell, and the small, ameboid or "tailed," actively wandering cell. Both types usually occurred in the same culture; they were either interspersed or, frequently, were in segregated groups of similar cells. The tailed cells were often arranged as small rosettes, or acini, their blunt ends directed toward the center and their tails distad.

The small, round, darkly staining thymocytes (pl. 40, fig. 1) appeared morphologically identical with lymphocytes and in general conform to descriptions of lymphocytes in carp blood by Dombrowski (1953). The cell was composed almost entirely of a large spherical nucleus in which the chromatin material appeared as small, darkly stained clumps, and a nucleolus was not visible. The nuclear membrane was comparatively thick and very chromophilic. In routine haematoxylin and eosin preparations, no cytoplasm was detected, and the nucleus seemed naked. However, with Mayer's haemalum, Wright's stain, and the Dominici stain, the cytoplasm was readily discernible as a very thin, light-staining rim about the nucleus. The cells varied in size, ranging in diameter from 2.40 μ to 3.84 μ, with a mean value of 2.98 ± 0.26. This mean was arrived at by the combining of the values of thymocyte diameters from several cultures. Statistically, such a procedure was sound, as the individual means were not significantly different. The data can be found in table 2. Mitotic figures in general were rare, but occasional cells in metaphase were observed.

In the active wandering stage, the basic thymocyte characteristics were retained (pl. 40, fig. 2). The shape of the cell, however, has

TABLE 2

COMPARISON OF THE DIAMETERS OF THYMOCYTE AND LYMPHOCYTE NUCLEI IN NORMAL AND MALIGNANT TISSUE FOR STATISTICAL SIGNIFICANCE

Tissue and Culture No.	Mean Nuclear Diameter in Microns	Standard Deviation	Standard Error	Significance[a]
Normal thymus (N-21)	2.94	0.19	0.02	
				2.1
Normal thymus (119b)	3.02	0.31	0.03	
				1.6
Thymus with compound E (78a)	2.95	0.31	0.03	
				0.8
Normal thymuses (N-21+119b)	2.98	0.26	0.02	
Tumor B (G-22)	3.83	0.87	0.09	
				0.3
Tumor B (G-5)	3.80	0.29	0.03	
				0.9
Tumor B with compound E (G-16)	3.76	0.32	0.03	
Normal thymus (119b)	3.02	0.31	0.03	
				18.5
Tumor B (G-5)	3.80	0.29	0.03	

[a] Calculated by the method employed by Rasquin and Rosenbloom (1954).

changed by virtue of the migratory activity. Owing to the small size of these thymocytes, it was not possible, with the ordinary light-field microscope, to study their movement in detail in the living cultures. In the stained specimens, however, under higher magnification, cytoplasmic processes indicative of ameboid movement were seen. The increased amount of pale-staining cytoplasm, in comparison to that found in the round thymocyte, may still be considered scant, and the nucleus to cytoplasm ratio is still very high. The nucleus was extended in length and often showed striking longitudinal striae, similar to those observed by Wassen (1915) in the frog thymus. It was usually pear-shaped and frequently unevenly forked at the tapered end, a phenomenon quite characteristic of these cells in our cultures. Often the tapered end was finely attenuated, and it is this cell that is referred to by Pfeiffer (1935) as the "tailed" thymocyte, and by De Bruyn (1944) as the "hand-mirror" type. In some of the cells, the chromatin material, as stained by haematoxylin, was more concentrated in the center of the nucleus, giving the appearance of a nucleolus. That this is not nucleolar material was substantiated by the fact that in methyl-green-pyronin-stained cultures, only the cytoplasm took up the pyronin, while the nucleus was stained by the methyl green to a greater or lesser extent, depending upon chromatin concentration, which does not necessarily indicate the absence of a nucleolus. Possibly the nucleolus, which ordinarily would be stained rose-pink by pyronin, is masked by the dense chromatin material. At no time were any dividing or degenerating forms seen. All stages between the round and the actively wandering forms were seen. With increasing age, however, the latter disappeared.

Scattered among the thymocytes were the larger, lighter-staining thymoblasts (pl. 40, fig. 3). These cells were much fewer in number and differed considerably from the thymocytes. They were round, oval, or occasionally pear-shaped. While the nucleus was larger, the chromatin particles were smaller and more widely dispersed, and a large central nucleolus, stained rose-pink by pyronin, was seen. The nuclear membrane was not so heavy nor so chromophilic as that of the thymocyte. Dark-staining, rather basophilic cytoplasm was present in greater quantity, although the

nucleus still occupied a major part of the cell. Mitotic figures were more numerous than in the thymocytes.

At no time were any of the cultures devoid of degenerating thymocytes. From the time that the cultures were made, all stages of disintegration were observed, ranging from pycnosis to fragmentation (pl. 40, figs. 4, 5). Many of the shrunken pycnotic nuclei and fragments thereof were phagocytized by the macrophages. It is interesting to note the persistence of some normal round thymocytes even in cultures as old as 14 days, although, in general, these cells have a very short life *in vitro*. Several cultures indicated a secondary migration of wandering ameboid thymocytes as well as thymoblasts after approximately 10 days *in vitro*, a condition also observed by Wassen (1915) in the frog thymus.

RED BLOOD CELLS: Those cultures that were made from a heavily vascularized fragment also showed numerous erythrocytes along with the initial migration of thymocytes. These cells, typically oval, with a centrally located, chromatin-rich oval nucleus, disappeared from the cultures within a few days, and considerable debris was observed in the medium.

EOSINOPHILIC WANDERING CELLS: As early as 27 hours after explantation, cells larger than thymocytes were occasionally seen in some cultures, wandering about quite rapidly in ameboid fashion (pl. 41, fig. 1). These were usually observed in cultures containing some sheets of epithelium, crawling about, either on or between the epithelial cells, or freely among thymocytes that were not trapped within the sheet. The frequency with which these wandering cells occurred increased with the age of the culture, but at no time were more than a dozen cells observed in any one culture. The cells appeared to move with the nucleus always in the trailing portion of the cell. Such movement was readily observed in those cells containing highly refractile cytoplasmic granules which facilitated distinction between cytoplasm and nucleus. When stained with haematoxylin and eosin, the granules were brilliantly eosinophilic and of uniform size. The eccentric nucleus was usually oval, often twice as long as it was wide, although occasionally in a more stationary cell it was spherical. In general, there is a strong similarity between this wandering-cell nucleus and a typical thymocyte nucleus. Both are approximately the same size, although the nucleus of the eosinophile was frequently lighter-staining with haematoxylin. The nucleus was often hyperchromatic, and the chromatin appeared as dark-staining clumps. In some cells, the resemblance to thymocytes was still greater in that the nucleus was forked at the distal end, a phenomenon quite common in the nucleus of the wandering thymocyte. It is quite probable, then, that thymocytes of different sizes were transformed into these eosinophilic wandering cells. Transformation cells of all stages have been observed, some bearing a close resemblance to the parent thymocyte (pl. 41, fig. 2). Similar transformation cells *in vitro* have been reported by Bloom (1937), using the rabbit thoracic duct, and Murray (1947), using the rabbit thymus.

Occasionally some ameboid cells were seen that were not granular, but that resembled the eosinophilic wandering cells in all other respects (pl. 41, fig. 3). Because of the homogeneity of the protoplasm, the nucleus could not be distinguished in the living cultures. The clear, homogeneous nature of the cells is somewhat obscured by other cells above or below, but just out of focus.

MACROPHAGES: Macrophages were first observed in the cultures at about the same time as were the thymocytes, within the first 24 hours after explantation. They were especially recognizable in those living cultures in which some of the pigmented integument had been included with the original thymus fragment. Under those circumstances, the macrophages were frequently large, rounded, and heavily laden with phagocytized melanin granules from the broken-down melanophores (pl. 41, fig. 4). In general, the size and shape of the macrophage were highly variable, depending upon the quantity of ingested material and the migratory state of the cell. In an active wandering condition, the cell body was elongated, with numerous irregular, protoplasmic processes. The cell moved ameboid fashion in and about the reticulum (pl. 41, fig. 5). With an increased amount of ingested material, however, the pseudopods were withdrawn, and the cell was more rounded. The nucleus, when visible, was often quite large,

oval, and pale-staining with haemotoxylin, with a somewhat darker nucleolus. It was frequently flattened against the cell membrane by the numerous cytoplasmic inclusions. Most often, in the heavily laden cell, the nucleus was obscured by the phagocytized material which included, in addition to pigment granules, pycnotic and fragmented thymocyte nuclei, shreds of cytoplasm, red blood cells, bits of plasma clot, and other debris. The number of macrophages increased with the age of the cultures. In those cultures in which trypan blue (1:100,000) had been included in the media, several rounded cells were seen laden with the blue dye within 24 hours (pl. 41, fig. 6).

MELANOPHORES: Frequently, when the thymus was extirpated, some of the pigmented integument was carried along. Usually the melanophores remained within the explant in an expanded state, but in some cases pigment-laden processes were seen extending into the zone of proliferation. Frequently the processes were lost, and the melanophores appeared as black clumps (pl. 41, fig. 7). In general, disintegration of these cells occurred within a few days, so that loose pigment granules were seen scattered throughout the cultures. Many granules were eventually phagocytized by macrophages.

The pigment cells in these cultures are similar to those originally described by Grand, Gordon, and Cameron (1941) and by Greenberg, Kopac, and Gordon (1956) for fish melanotic tumors in tissue culture.

EPITHELIAL RETICULUM: Epithelial reticular elements appeared in the majority of cultures between 36 and 48 hours after explantation. However, in some cultures, epithelium was seen within the first 24 hours. The early growth was usually a single layer of flat cells adherent to the cover slip. Figure 1 of plate 42 shows the beginning of epithelial proliferation as a small continuous sheet in a 48-hour culture. In the living state, the cells appeared to have a clear, round to oval nucleus, surrounded by a large quantity of cytoplasm, often containing refractile granules similar to those observed by Tschassownikow (1927) and Murray (1947) in cultures of rabbit thymus, and by Wassen (1915) in cultures of frog thymus. With age, the number of granules increased. In addition to growing directly on the cover slip, the epithelium was frequently seen proliferating on the surface of the plasma clot, and occasionally within the deeper layers of the clot, so that in some cultures, several layers of cells were observed.

The epithelium grew in a variety of forms, the most common of which was the typical, single-layered, tight, compact sheet of polygonal cells. Figure 2 of plate 42 shows such proliferation in a 72-hour culture. With higher magnification (pl. 42, fig. 3) the syncytial arrangement of the cells can be seen; in general, cell borders were not distinguishable. In some cultures, there was a separation of the cells so that individual cell boundaries were visible. The growing edge of the sheet was usually smooth and regular, although frequently the peripheral cells were more loosely arranged, and irregular processes were observed. Some cells broke away from the sheet and remained as individuals or anastomosed with one another, a situation described by Levi (1934) as typical of most epithelial growth *in vitro*. These "stragglers" were usually more spindle-like and possessed several irregular cytoplasmic processes (pl. 42, fig. 4), which made it difficult to determine whether these cells were epithelium or connective tissue. Fischer (1922) described similar fibroblast-like cells at the periphery of well-grown epithelial cultures.

Many liquefied areas, probably the result of a proteolytic effect of the epithelium on the fibrin clot, were observed, so that occasionally the sheet of cells appeared to be full of holes. Frequently, the liquefaction of the clot caused a mechanical displacement of the cells which had been growing over that area. There was a retraction of the entire sheet, and the cells were stretched so taut that they somewhat resembled the connective tissue spindle cells. Liquefaction of the medium appears to be quite diagnostic in cultures of lymph nodes involved in Hodgkin's disease (Grand, 1949; Rottino and Hollender, 1949a).

In some cultures, the epithelium proliferated as strands or cords of cells anastomosing with other strands or cords (pl. 43, figs. 1–3). The cells comprising the strands were usually aligned parallel to one another, actually in contact along the entire length of the cell. At

times, there were so many anastomosing cords that the over-all effect was that of a lace-like sheet.

In several cultures, the epithelium grew as a tongue of syncytial cells with a smooth margin. Thymocytes were frequently clustered about the periphery of the tongue (pl. 43, figs. 4, 5).

Frequently, epithelial "islands" were formed (pl. 44, fig. 1). These were the result of a condensation of cells in a restricted area, detached from the original explant. Some may have been formed by repeated divisions of an individual cell; others may have parted as a cluster from the intact sheet, probably because of liquefaction of the medium. The islands varied in size and were usually irregular in outline. Occasional peripheral cells had long fine processes which anastomosed with similar processes of other cells.

Of interest are the so-called "pearl" formations seen in several older cultures (pl. 44, figs. 2, 3). In the midst of a sheet of epithelium, a group of flattened cells was observed arranged in concentric rings, vaguely reminiscent of the Hassall's bodies in higher vertebrates. Only occasionally was debris seen at the core. In one case, a single dividing cell was noted.

Also characteristic of the epithelium, especially in one-week-old cultures, are cystic spaces within the sheet. These appeared as small holes, either individually or in groups (pl. 44, figs. 4, 5), with flattened nuclei at the periphery of the cells. Coalescence of some of the smaller spaces to form larger ones was occasionally seen.

One to several of the above forms of epithelium were usually seen per culture, except in some of the thymus cultures from very young fish in which no reticular cells were evident. In general, the epithelial proliferation was rapid and extensive, so that, within 48 to 72 hours, the zone of outgrowth in some cultures was as much as five times the diameter of the original thymus fragment. Mitotic figures were not seen in all the actively proliferating cultures. When present, however, they were abundant, and all stages of mitosis were clearly defined in the stained specimens. Normal and degenerating thymocytes, in addition to other migrating cells, were seen scattered throughout the entire epithelial sheet.

In haematoxylin and eosin-stained cultures, the large quantity of eosinophilic cytoplasm of these epithelial cells appeared to be pale and finely granular. Eosinophilic inclusions, as well as vacuoles, were frequently observed, which gave a frothy appearance to some of the cells. Some cells were phagocytic, and entire and fragmented nuclei, presumably from thymocytes, were seen in the cytoplasm. Trowell (1949b) reported similar intracellular thymocytes in the reticular cells in hanging-drop cultures of rat thymus. The vesicular nucleus was usually round to oval, with little chromatin. In addition, a variety of bizarre-shaped nuclei was also observed side by side with seemingly normal nuclei. Some intranuclear vacuoles were noted. The nucleolar pattern was quite variable, as to both number and shape. Even in binucleate cells, the patterns were not always the same for both nuclei. While the majority of the nuclei were of approximately the same dimensions (diameter, 14.0 μ), all sizes ranging from 7.2 μ to 39.6 μ were seen. When the nucleus is oval, these values refer to the longer diameter. This polymorphous condition of the epithelial cells was also observed in roller-tube cultures of rabbit thymus described by Mendelsohn (1954). Figure 1 of plate 45 shows both typical and irregularly shaped nuclei side by side. In plate 45, figure 2, the extreme variation in nuclear size is especially evident. Again, an irregularly shaped nucleus can be seen close to a seemingly normal nucleus, which is in anaphase. These bizarre nuclei assumed a variety of shapes. Some were lobulated (pl. 45, fig. 3); others had one to several buds. Occasionally, the nucleus appeared to be made up of a string of basophilic fragments, not always of the same size and frequently connected to one another. Most of these fragments contained nucleoli. It is quite probable that many such irregularities were the result of amitotic divisions as seen in plate 45, figure 4. While these occurrences were more frequent in older cultures, some bizarre nuclei were seen wherever epithelial sheets had proliferated.

Binucleated epithelial cells were fairly common in the thymus cultures and may have oc-

curred even more frequently than is apparent. However, owing to the syncytial nature of the epithelial cells and the indistinguishable cell borders, it was often difficult to determine whether or not two particular nuclei belonged to the same cell. Consequently, only those cells in which the nuclei abutted were considered as true binucleate cells (pl. 46, fig. 1). In general, both nuclei were typically epithelial and identical in size and nucleolar configuration, although dissimilar ones were seen in which the nucleolar number differed. Because nuclear size is variable in epithelial cells in general, one cannot state with certainty that the combined nuclei were equivalent in volume to one large nucleus. It seems more likely that each of the pair was of normal size. Some cells were noted with three and four such identical nuclei. The binucleate condition of these epithelial cells prevailed in cultures of all ages and appeared with the onset of epithelial proliferation.

In older cultures, usually of one week or more, a different type of binucleation and subsequent multinucleation was observed. These cells were usually seen farther from the explant in the older regions of the epithelial sheet. These binucleate cells, probably forerunners of multinucleated giant cells, were readily distinguishable from the typical binucleate epithelial cell described above. Individual cell boundaries were clearly demarcated. The nuclei were smaller and more basophilic than those of the neighboring epithelial cells. The cells shown in figures 2 and 3 of plate 46 differed from those shown in figures 4 to 6 in that the latter contained considerable quantities of phagocytized material. It seems likely that the five-nucleated cells depicted in figure 3, developed from such a binucleate cell as seen in figure 2 of the same plate, and the multinucleated cells of figures 5, 6, and 8 probably arose from a cell similar to that shown in figure 4. While the following description is for the largest and most diagrammatic of the multinucleated cells (pl. 46, fig. 8), the same general scheme obtained for the others as well. As seen in figure 7 of plate 46, this cell lay at a considerable distance from the explant, in the zone of proliferation in a 14-day thymus culture. It was extremely large, flat, and somewhat rectangular in shape. Length and width measurements, made through the center of the nuclear ring, indicated that the cell was 127 μ long by 94 μ wide, and the nuclei occupied an area approximately 84 μ by 67 μ. A finely granular central core was conspicuous, around which all the nuclei, with the exception of two, were located. The nuclei were vesicular, contained one centrally located nucleolus, and in general were smaller and more basophilic than those of neighboring epithelial cells. They were mostly oval, but occasionally a spherical one was also seen. It is interesting to note that many of the oval nuclei were finely attenuated in the direction of the central core, which suggests a recent amitotic division. In this cell, 37 nuclei were counted, but other cells were seen in which there were fewer nuclei, although the same distribution of cellular components existed. Often the nuclei formed only a horseshoe rather than a complete ring, and in many instances the cells were elongated, as seen in plate 46, figure 6. The cytoplasm peripheral to the ring of nuclei was less dense and therefore lighter staining. It contained vacuoles (possibly fat globules) and much phagocytized material, including nuclei, cytoplasmic fragments, and other non-distinguishable debris. Throughout the central core, nuclear area, and the zone containing phagocytized material, there was a fine scattering of pigment granules which probably came from disintegration of the melanophores in the skin covering the gland during growth in culture. Beyond this area, the ectoplasm was again finely granular and quite dense. The cell outline was irregular and frequently was composed of very short processes by which the cell seemed to be attached to the cover slip. This type of giant cell is very similar to that described by Langhans (1868) as associated with tuberculosis and now commonly referred to as the "Langhans" giant cell. It is interesting to note that Lewis and Webster (1921) reported a typical multinucleated giant cell in an eight-day culture of a tuberculous human lymph node which is almost identical with that described above and shown in plate 46, figure 8.

FIBROBLASTS: Owing to the inherent nature of the thymus to involute with age, cultures made of the glands of younger fish were different from those of older fish. The most obvious difference was the appearance of fibroblasts in

cultures of fish four and one-half months or older. As part of the involution phenomenon, there is an increase in the elements of connective tissue in both the capsule and the subcapsular portions of the parenchyma as well as around the capillaries. Hafter (1952) reported that these changes occur in the thymus of *Astyanax* at about 26 weeks of age. During the early stages of our experiments, most of the fish used were between six and eight months old. For all subsequent studies, however, two-month-old fish were used to better advantage. No fibroblasts were seen in this latter group. On the whole, only 10 per cent of the cultures showed signs of fibroblast growth, and at no time could this growth be referred to as luxuriant.

Fibroblasts made their earliest appearance in several cultures 48 hours after explantation, although for the most part, a longer time (at least 72 hours) was required (pl. 47, figs. 1, 2). The first cells to leave the explant proliferated in a radial manner, that is, perpendicular to the edge of the explant, usually penetrating the sheet of epithelium. Such proliferation is typical of fibroblastic growth. In many cases a tangential type of proliferation also occurred. Here the fibroblasts were seen layered parallel to the edge of the fragment and, in effect, almost "encapsulating" it.

The cells may be described as long, fine spindles in which the widest diameter of the cytoplasmic area rarely exceeded that of the nucleus. The cytoplasm was outstretched to form very long processes, often forked at the distal end. Sometimes rebranching was also observed, reminiscent of dendrites. Quite frequently, the processes were so long and fine that the ends were not visible. The oval nucleus was long and narrow, usually about five times as long as it was wide. In comparison with the epithelial nucleus, it was more basophilic and contained more chromatin. One to three nucleoli were present. Often the nucleolus was elongated to conform to the shape of the nucleus. Usually the cells were individually arranged; however, many regions were noted in which the fibroblasts were lined up end to end, giving the appearance of a series of nuclei without visible cell boundaries. Sometimes the cells were side by side, almost sheet-like. Mitotic figures were quite frequent. With increased proliferation, the cells grew in several directions, sometimes anastomosing with one another to form a mesh. Figure 3 of plate 47 shows an intermingling of fibroblasts with epithelial cells.

Histochemical Determinations

Nucleic Acids: Nucleic-acid determinations on normal thymocytes indicated a very heavy accumulation of desoxyribonucleic acid (DNA) in the nucleus. The cytoplasm of the round thymocytes appeared as a thin ring of ribonucleic acid (RNA) immediately around the nucleus. In the tailed and ameboid forms, RNA was present throughout the irregularly shaped cytoplasmic area. No nucleoli were observed in these cells. In contrast, the thymoblasts showed a greater concentration of RNA in the cytoplasm and a less dense accumulation of nuclear DNA with a central nucleolus.

Epithelial nuclei were weakly positive for DNA. There seemed to be little chromatin dispersed throughout the large nucleus. The granular cytoplasm, as well as the honogeneous-looking nucleoli, also was weakly positive for RNA.

The cytoplasm of macrophages, at times hardly visible on account of phagocytized inclusions, frequently showed dense accumulations of DNA which may be attributed to the mass of ingested nuclear material. In those macrophages containing little or no in-

TABLE 3

Nucleic Acid Determinations

Site of Reaction	Occurrence of Specific Nucleic Acid	
	DNA	RNA
Thymocyte nucleus	+	−
Thymocyte cytoplasm	−	+
Thymoblast nucleus	+	−
Thymoblast nucleolus	−	+
Thymoblast cytoplasm	−	+
Epithelial nucleus	Weakly +	−
Epithelial nucleolus	−	Weakly +
Epithelial cytoplasm	−	Weakly +
Macrophage nucleus	+	−
Macrophage cytoplasm	−	+

clusions, the cytoplasm was intensely positive for RNA. The nucleus showed a heavy concentration of DNA, when not obscured by the cytoplasmic inclusions.

The distinction between the two nucleic acids was corroborated with the use of ribonuclease which digested those substances that stained red with pyronin. The methyl green-staining DNA was resistant to digestion with ribonuclease. Figure 4 of plate 47 shows a typical culture stained with methyl green-pyronin. When contrasted with figure 5 of plate 47, in which the culture had been pretreated with ribonuclease, it is apparent that the cytoplasm is no longer discernible. Table 3 summarizes the nucleic acid determinations.

GLYCOGEN AND MUCOPOLYSACCHARIDES: Several histochemical tests were applied to determine the nature of inclusions sometimes seen in epithelial cells.

Bauer-Feulgen-positive inclusions were seen in some epithelial cells (pl. 48, figs. 1, 2). Predigestion with saliva did not alter the stainability of the Bauer-Feulgen-positive material, which precludes the possibility of the existence of glycogen in the thymus cells *in vitro*, and indicates the presence of another carbohydrate-containing substance, possibly a mucopolysaccharide.

The frequency and location of inclusions that were Periodic Acid-Schiff (PAS) positive approximated quite closely those of Bauer-Feulgen-positive material (pl. 48, fig. 3). Control cultures, pretreated with saliva, continued to show the same staining reaction with PAS, thereby supplying additional evidence for the absence of glycogen and the possible presence of a mucopolysaccharide.

The reaction to Best's ammoniacal carmine solution was negative, which is another and classic indication of the lack of glycogen in the cells of the thymus.

In consideration of the above reactions, the presence of a mucin was suggested. Two other techniques (Mayer's mucicarmine and Alcian blue) were therefore employed to establish more conclusively the presence of mucin in these cells. With both techniques for mucin, positive reactions were obtained, which were similar in nature and distribution to those described above for the Bauer-Feulgen and PAS techniques. Figures 4 and 5 of plate 48 show several cells containing inclusions that were Alcian blue positive.

On the basis of the results obtained for the various carbohydrate determinations, it seems highly probable that some of the epithelial cytoplasmic inclusions are mucopolysaccharide in nature, whereas none of the inclusions contains glycogen. The fact that they were stainable with Alcian blue further indicates an acid rather than a neutral mucopolysaccharide.

LIPIDS: A dense accumulation of intensely stained black droplets was seen in the cytoplasm of the epithelial cells when stained with Sudan Black B and counter-stained with Mayer's carmalum (pl. 49, fig. 1). A high concentration of lipid material in the epithelial cells is indicated by these results.

The appearance of brick-red rings, arcs, or irregularly shaped globules in the cytoplasm of the epithelial cells in cultures stained with Sudan IV suggests the presence of neutral fats (pl. 49, fig. 2). Some of these positively stained inclusions were probably located around other droplets (perhaps phospholipid).

Baker's Acid Hematein technique was employed to determine whether or not any phospholipids were present in the epithelial cells. Granules which stained blue-black were irregularly distributed throughout the cytoplasm (pl. 49, fig. 3). These granules were not uniform in size and for the most part were smaller and fewer in number than the Sudan Black positive material. Because they did not stain following pyridine extraction, a phospholipid is suggested. Some, but not all, of the degenerating thymocyte nuclei which were free or had been ingested by macrophages gave a positive reaction with the acid hematein technique. Occasionally, one or two small positive granules were seen at the periphery of a normal thymocyte. Despite prior extraction with pyridine, some of the degenerating thymocytes still were stained with acid hematein. In addition, the nucleoli of the epithelial cells, which did not stain with acid hematein alone, did stain following pyridine extraction (pl. 49, fig. 4). Possibly the degenerating thymocyte nuclei and the epithelial nucleoli contained bound phospholipids which were not readily extractable with pyridine, rather than

PLATES 39–51

PLATE 39

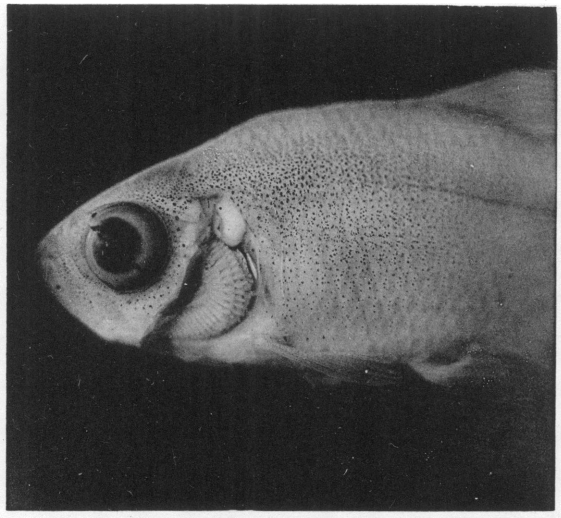
1. Photograph of a 14-week old *Astyanax mexicanus*, showing the thymus in the superior corner of the branchial cavity. The operculum and lens were removed after fixation. Courtesy of E. Hafter. ×5.

2. Thymus culture, one day after explantation, showing persistence of vascularization of the original fragment. Living. ×100.

3. Thymus culture one day after explantation, showing halo-like migration of thymocytes. Living. ×100.

4. Thymus culture five hours after explantation, showing delimited thymocyte migration. Living. ×70.

5. Thymus culture three days after explantation, showing endothelial tube-like tongue of thymocytes. Living. ×100.



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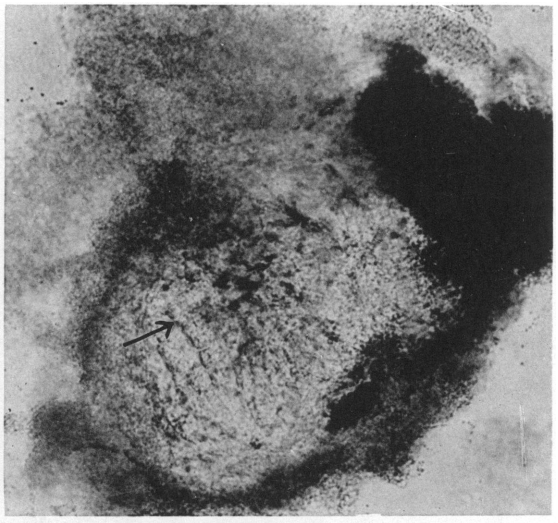

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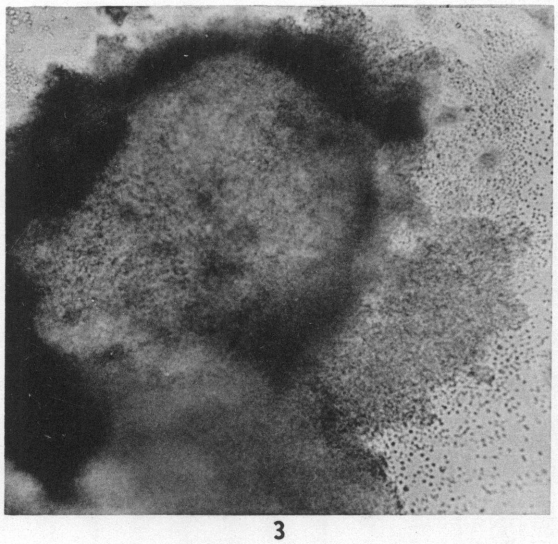

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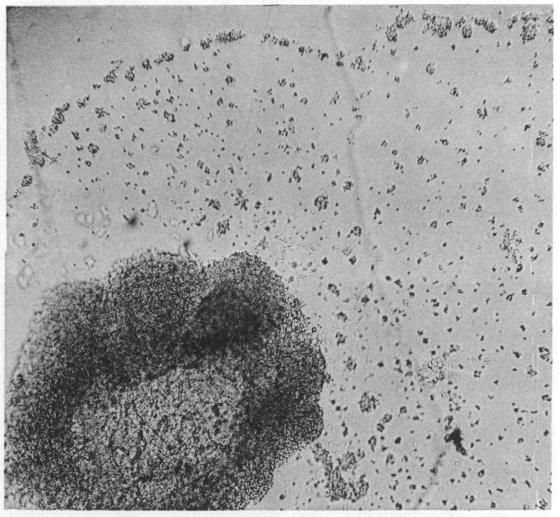

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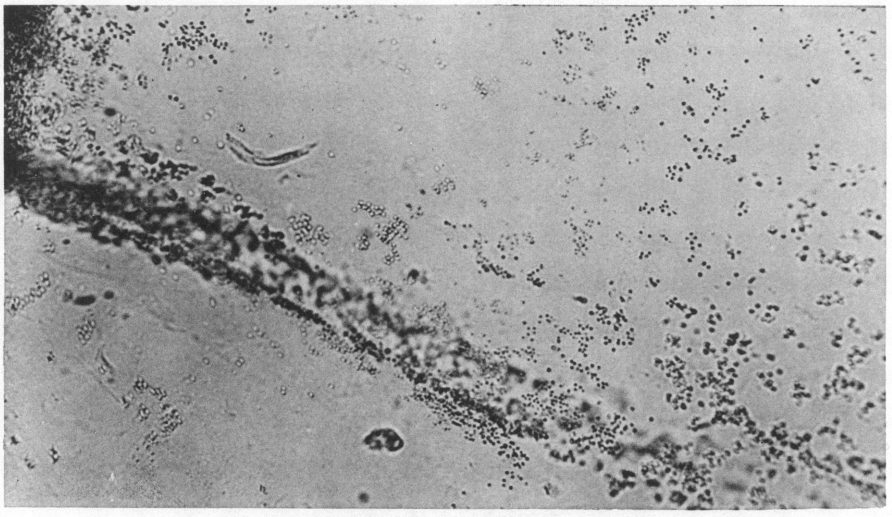

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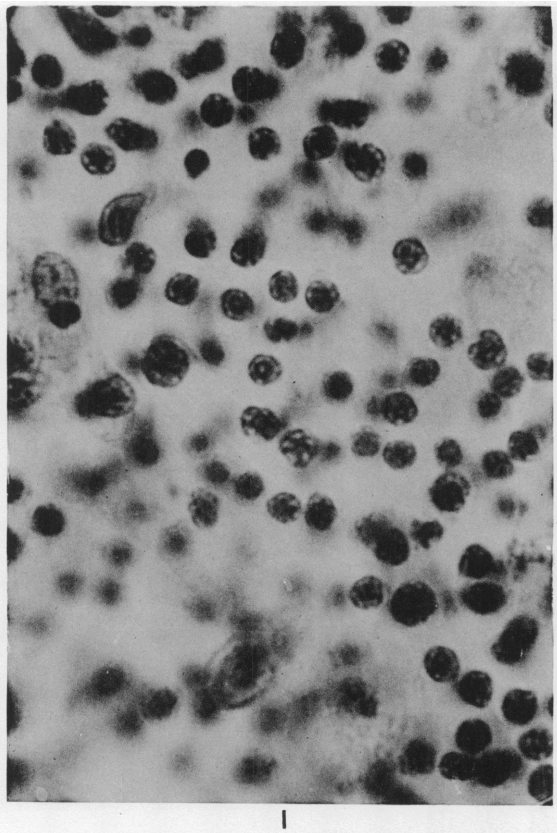

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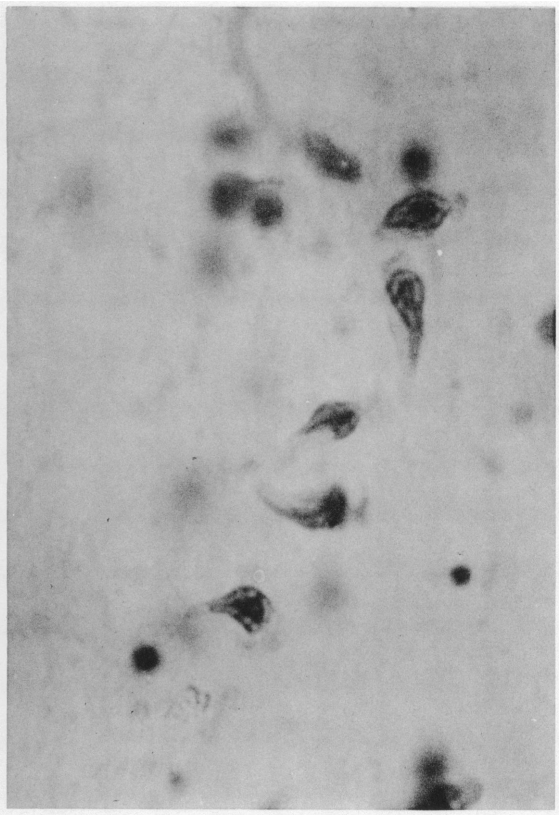

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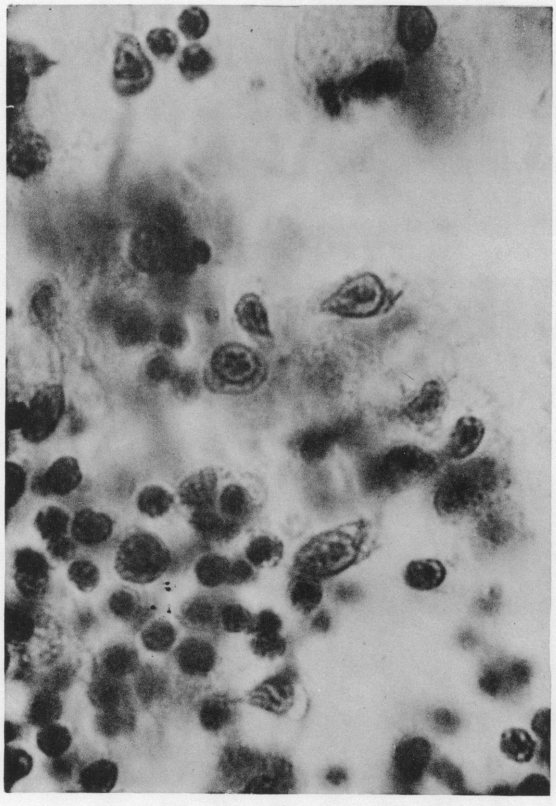

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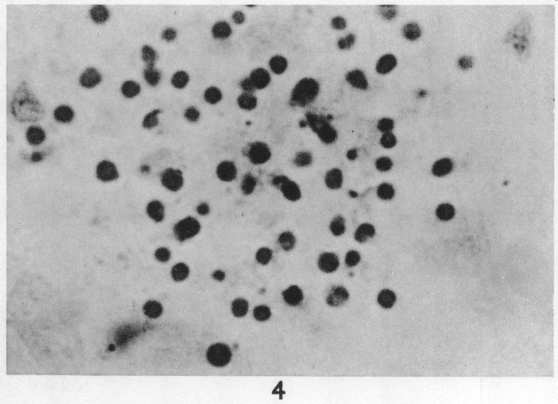

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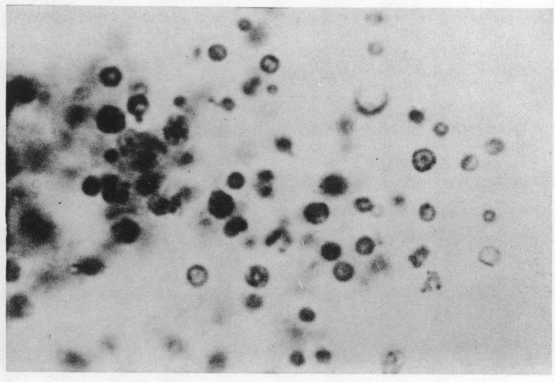

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PLATE 40

1. Photomicrograph of normal, healthy, round thymocytes in a two-day thymus culture. Harris' haematoxylin and eosin. ×1250.
2. Normal, healthy, tailed thymocytes in a one-day culture of thymus. Harris' haematoxylin and eosin. ×1250.
3. Thymus culture, two days after explantation, showing thymoblasts. Harris' haematoxylin and eosin. ×1250.
4. Pycnotic thymocytes in a one-day culture of thymus. Harris' haematoxylin and eosin. ×1250.
5. Degenerating thymocytes, showing ring forms, lunar forms, and fragmented nuclei. Harris' haematoxylin and eosin. ×1250.

PLATE 41

1. Small, eosinophilic, wandering cell in a 14-day thymus culture. Harris' haematoxylin and eosin. ×560.

2. Thymus culture five days after explantation, showing transforming thymocytes. Note typical thymocyte nucleus. Harris' haematoxylin and eosin. ×1250.

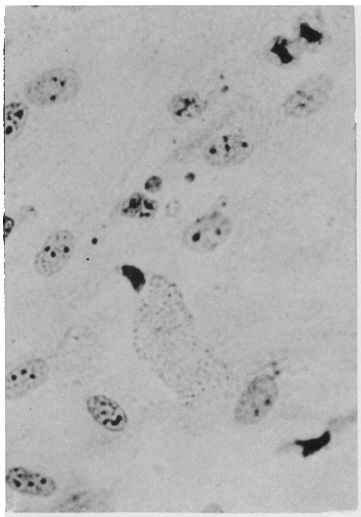
3. Clear ameboid wandering cells in a three-day culture of thymus. Living. ×560.

4. Macrophages heavily laden with melanin granules in a three-day thymus culture. Note rounded resting form of the cell. Living. ×560.

5. Macrophages with some ingested melanin granules in a five-day thymus culture. Note ameboid shape of this partially laden cell. Harris' haematoxylin and eosin. ×650.

6. Two-day thymus culture, showing macrophage containing ingested trypan blue (1:100,000). Living. ×650.

7. Melanophores within the explant of a seven-day thymus culture. Harris' haematoxylin and eosin. ×125.



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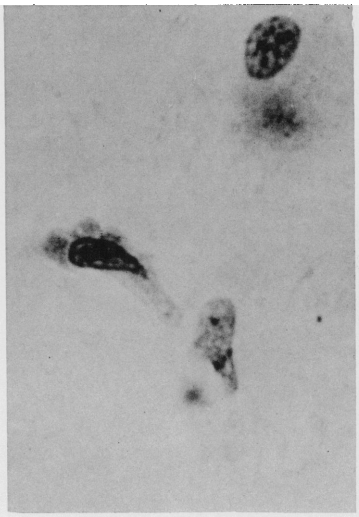

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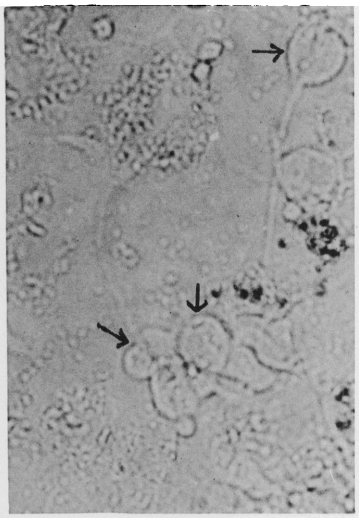

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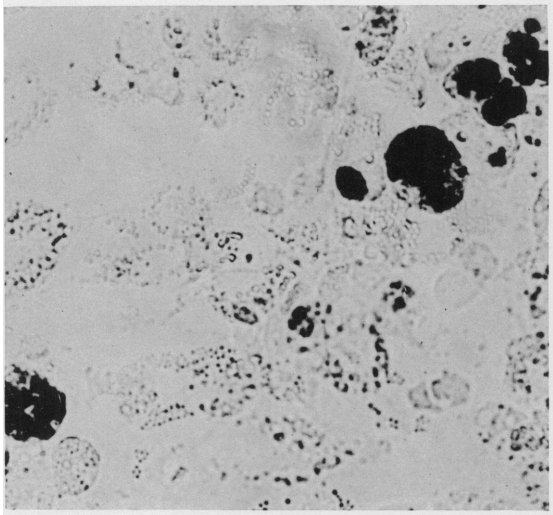

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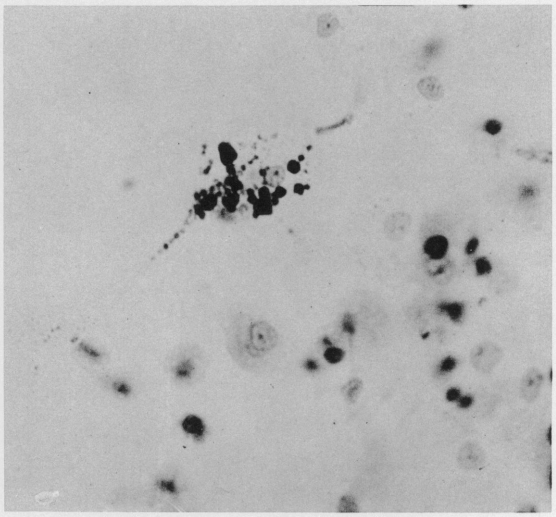

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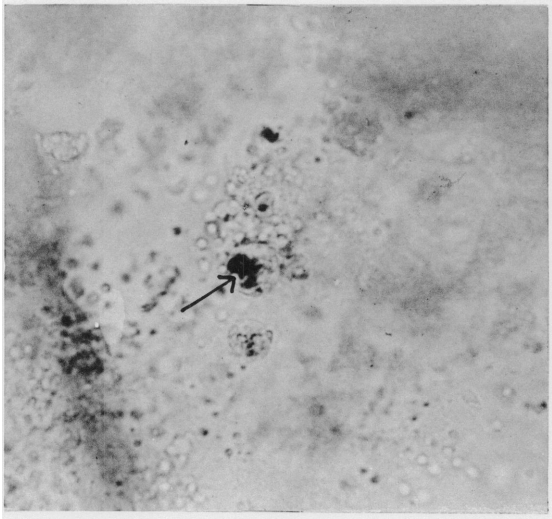

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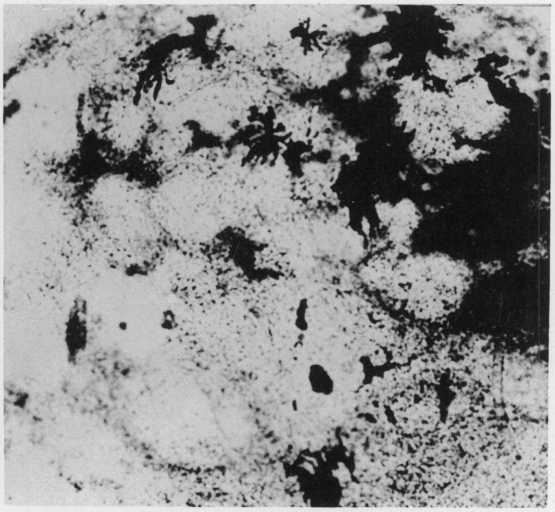

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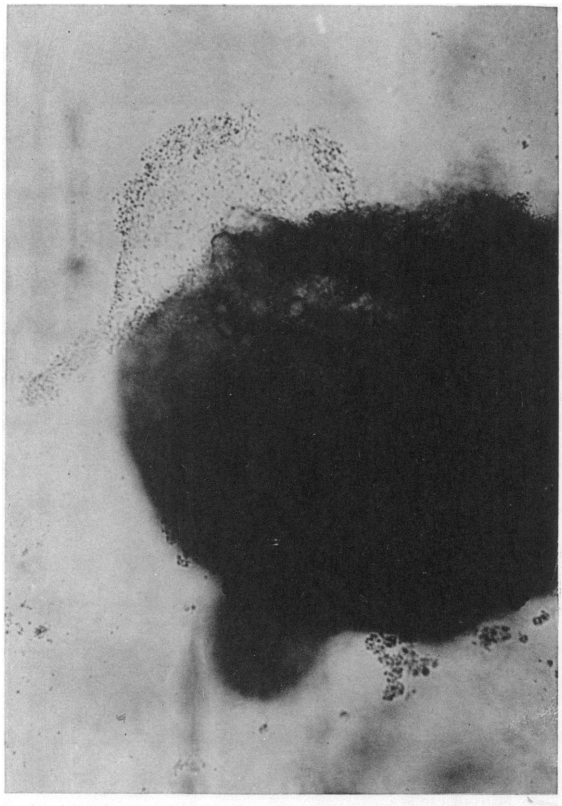

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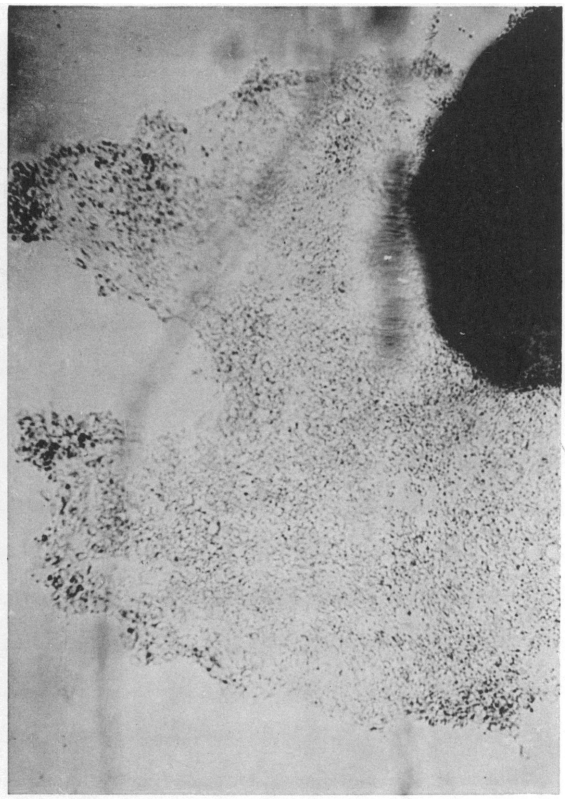

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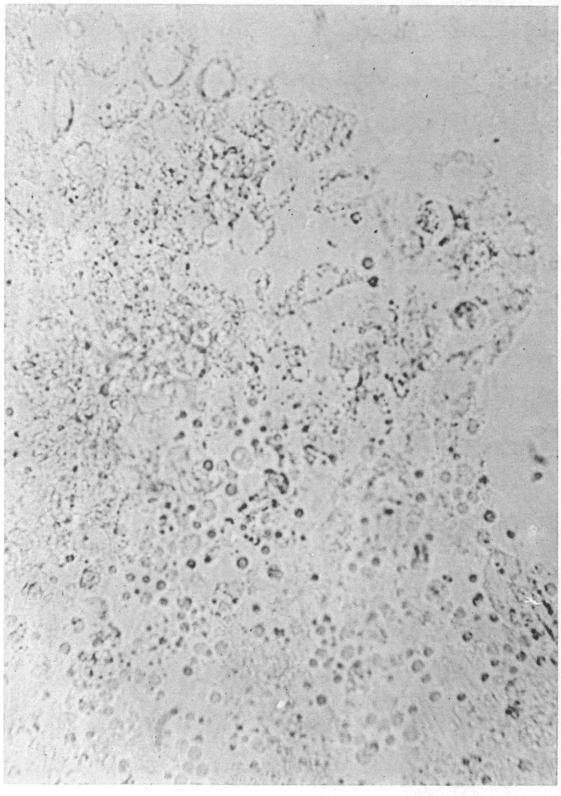

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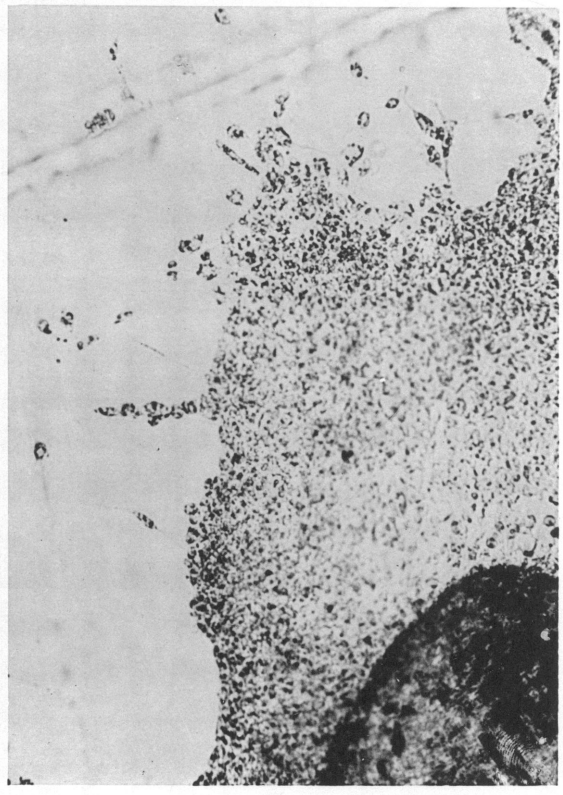

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PLATE 42

1. Two-day thymus culture, showing beginning of epithelial proliferation in the form of a continuous sheet. Living. ×100.

2. Thymus culture three days after explantation, showing extensive epithelial sheet. Note smooth, continuous, growing edge. Living. ×100.

3. Higher magnification of a typical epithelial sheet in a three-day thymus culture. Living. ×650.

4. Three-day thymus culture, showing epithelial sheet proliferation. Some peripheral cells ("stragglers") break away from the sheet and either remain as individuals or anastomose with one another. Living. ×106.

PLATE 43

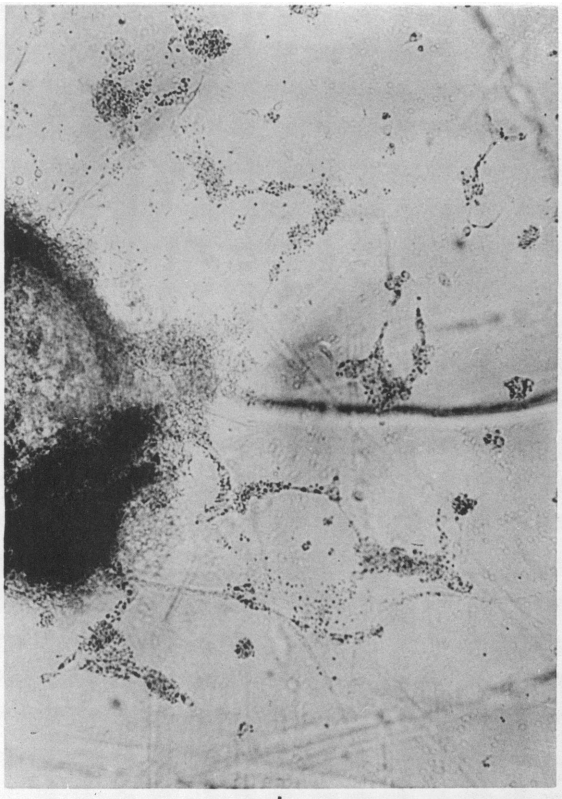
1. Two-day thymus culture in which the epithelium has proliferated as strands. Living. ×100.

2. Higher magnification of the culture shown in 1, to show anastomoses of the individual cells or strands. Living. ×650.

3. Same culture as shown in 1 and 2, four days after explantation. Note further proliferation of anastomosing strands of cells rather than a continuous sheet. Living. ×100.

4. Two-day thymus culture, showing epithelial proliferation in the form of a tongue. Living. ×100.

5. Same as 4, at higher magnification. Note smooth margin of tongue as well as clustering thymocytes. Living. ×650.



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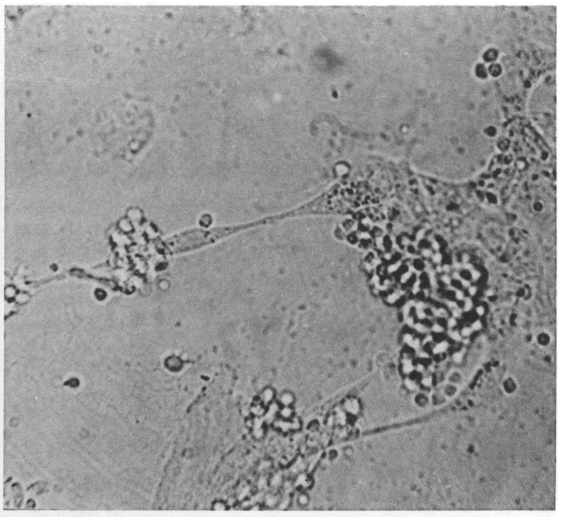

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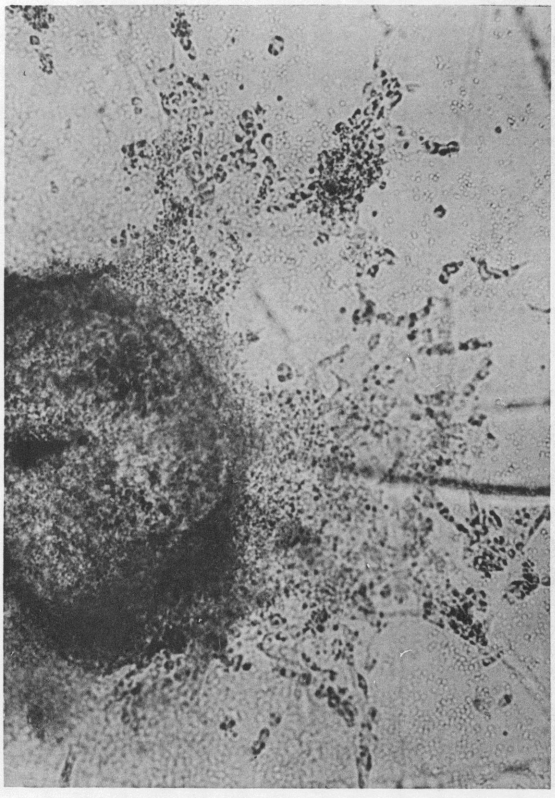

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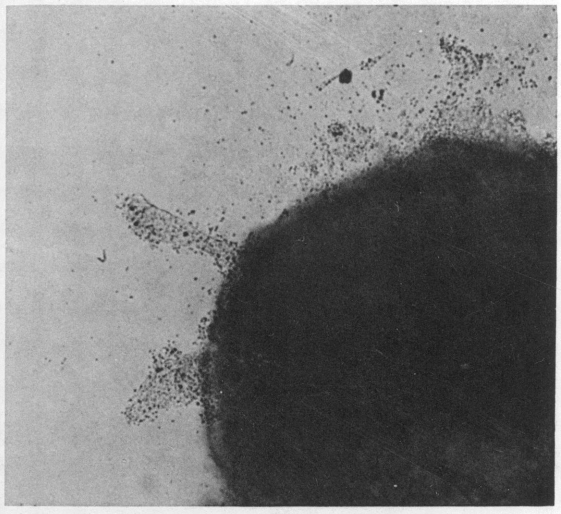

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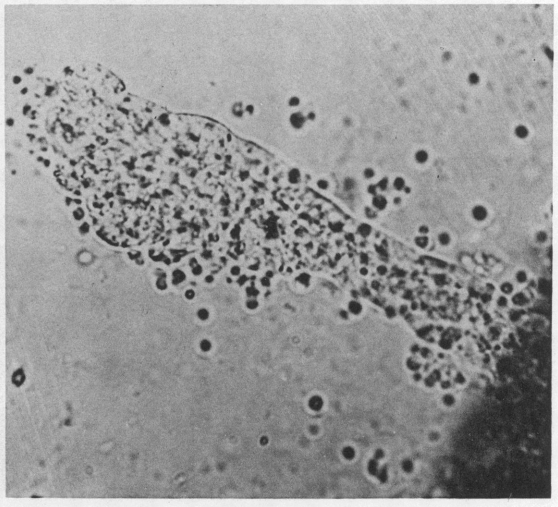

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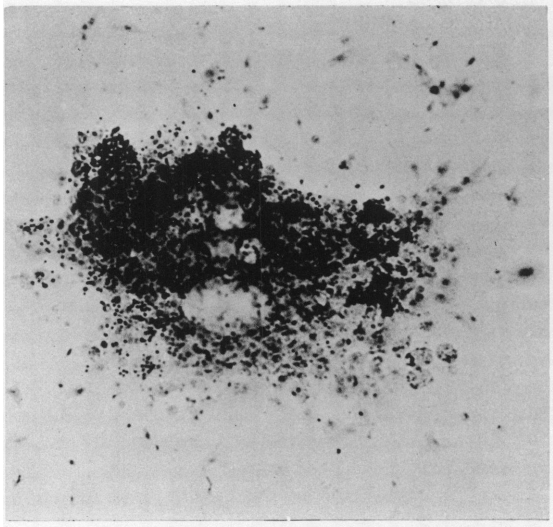

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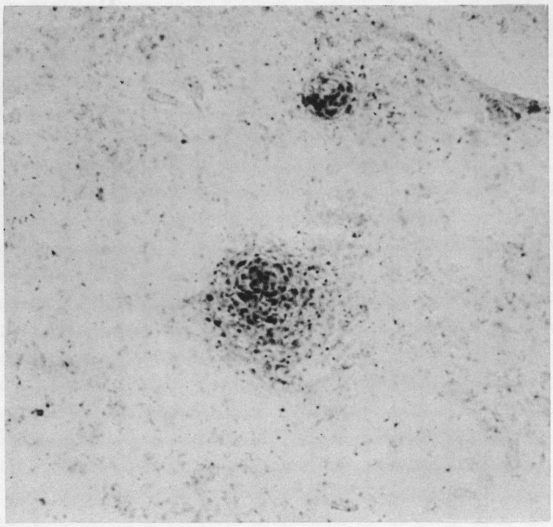

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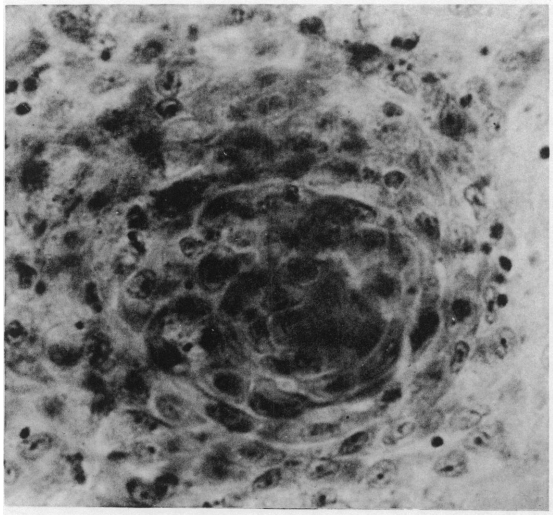

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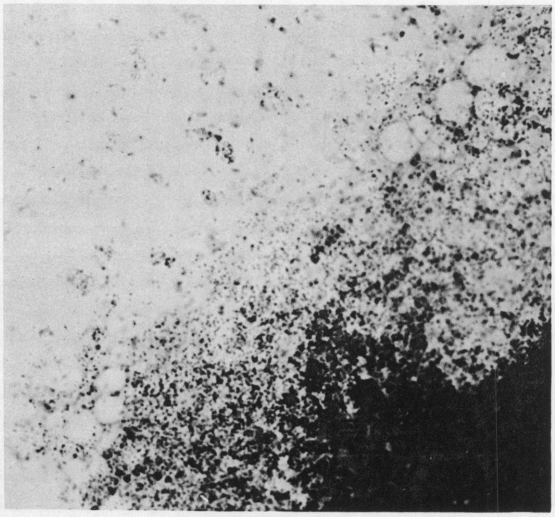

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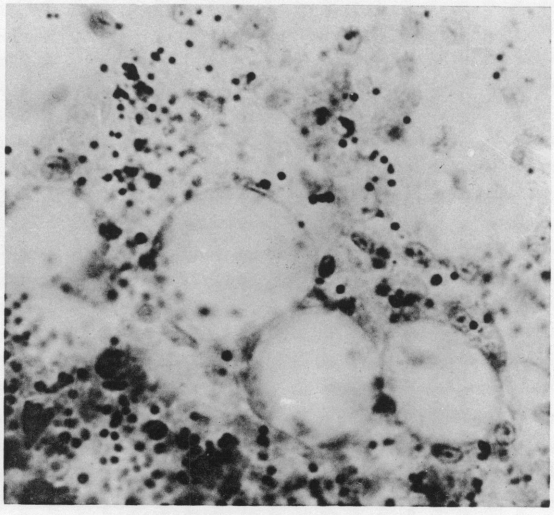

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PLATE 44

1. Thymus culture, five days after explantation, showing an epithelial island. Original explant is not within field of photograph. Harris' haematoxylin and eosin. ×150.

2. Thymus culture eight days old, showing a sheet of epithelium with two "pearl" formations. Harris' haematoxylin and eosin. ×125.

3. Higher magnification of epithelial "pearl," to show concentric arrangement of the cells. Harris' haematoxylin and eosin. ×540.

4. Seven-day thymus culture, showing epithelial sheet with cystic spaces. Harris' haematoxylin and eosin. ×125.

5. Higher magnification of cystic spaces. Note flattened nuclei at periphery of cells. Harris' haematoxylin and eosin. ×540.

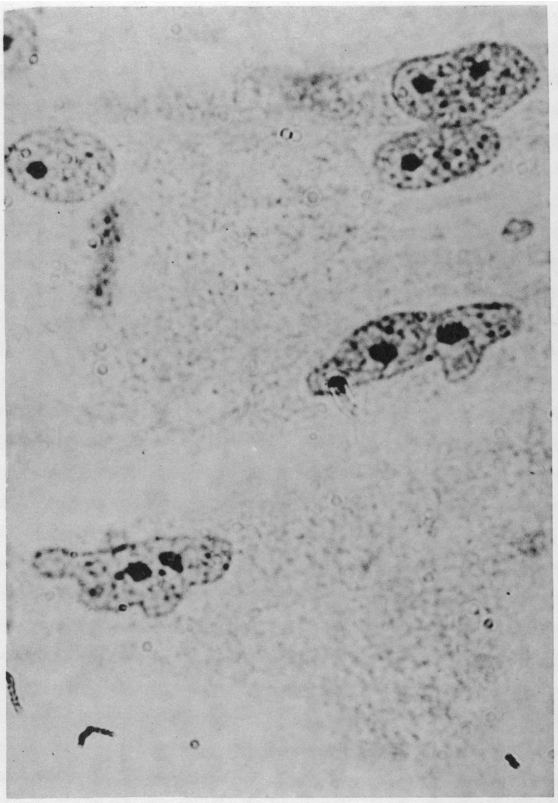
PLATE 45

1. Epithelial cells in a 14-day thymus culture, showing typically round to oval vesicular nuclei as well as bizarre-shaped nuclei. Harris' haematoxylin and eosin. ×1280.

2. Epithelial cells in a 14-day thymus culture, showing extreme variation in the size and shape of nuclei. Note normal-appearing nucleus in anaphase. Harris' haematoxylin and eosin. ×1280.

3. Bizarre epithelial nucleus in a 10-day thymus culture. Note lobulation. Harris' haematoxylin and eosin. ×1280.

4. Epithelial nucleus of a 14-day thymus culture undergoing apparent amitosis. Note dividing nucleolus. This may be the mechanism whereby the nucleus fragments or becomes lobulated. Harris' haematoxylin and eosin. ×1280.



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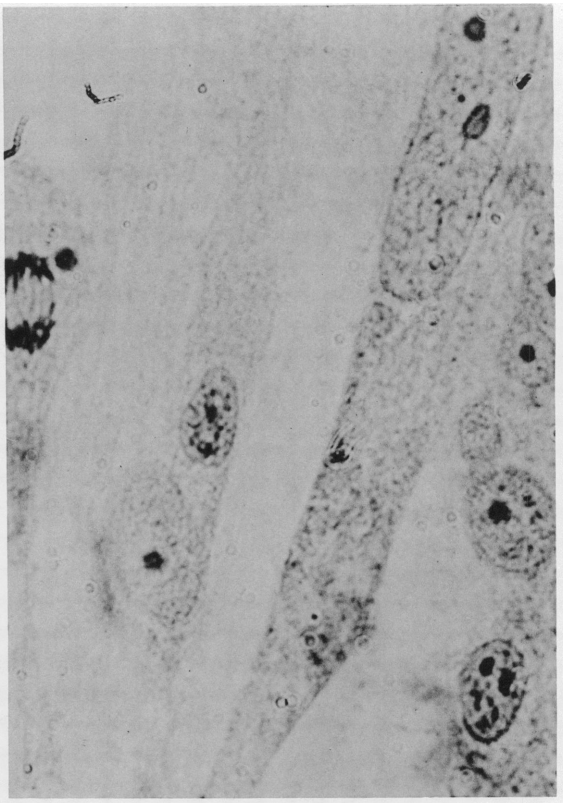

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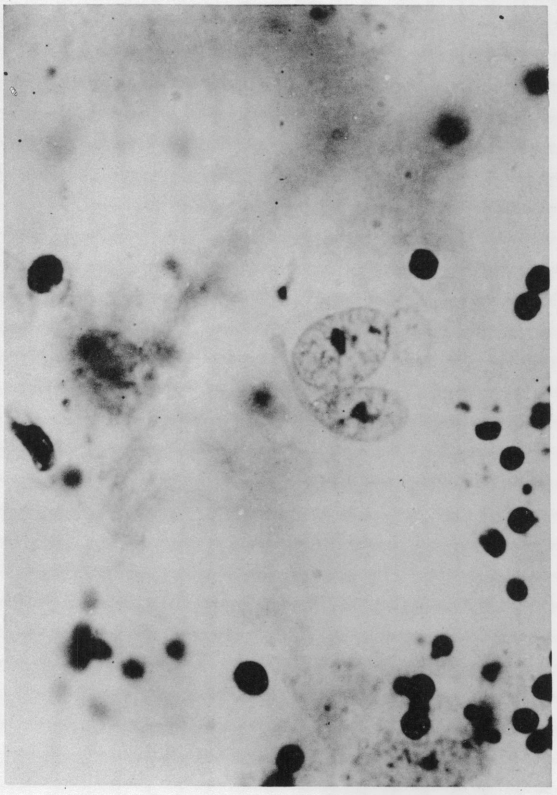

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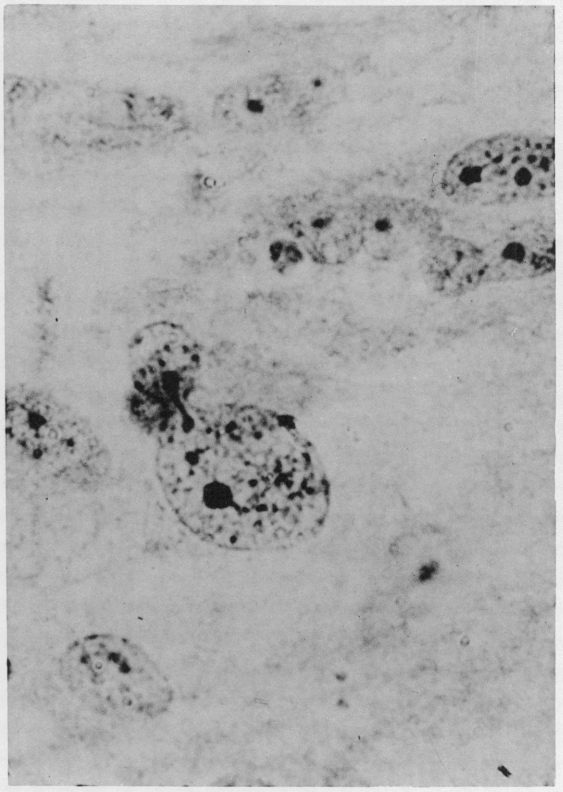

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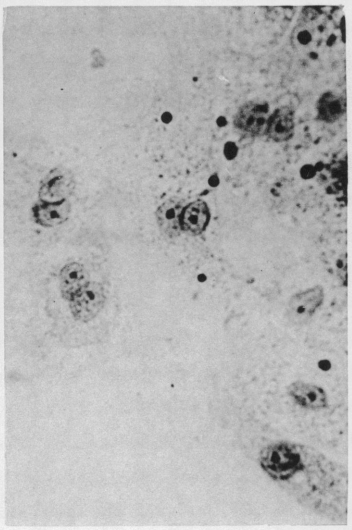

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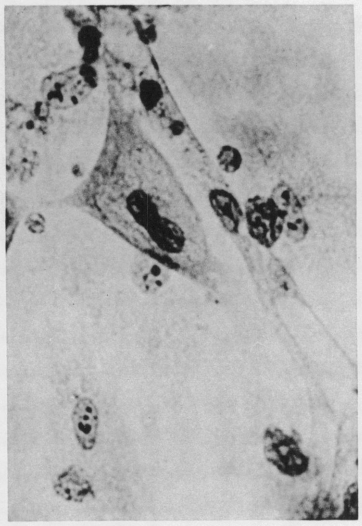

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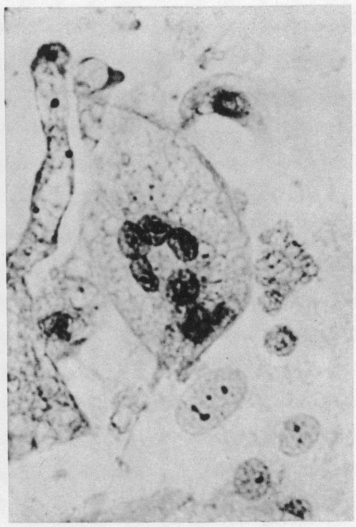

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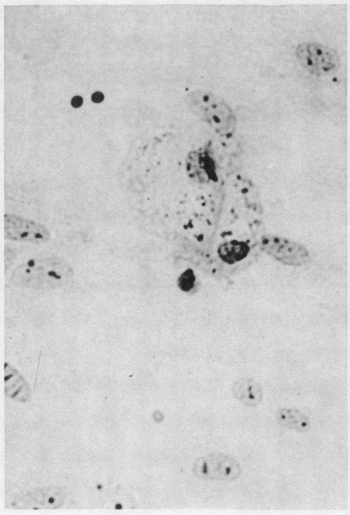

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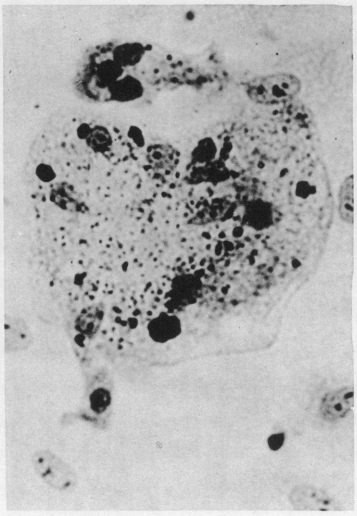

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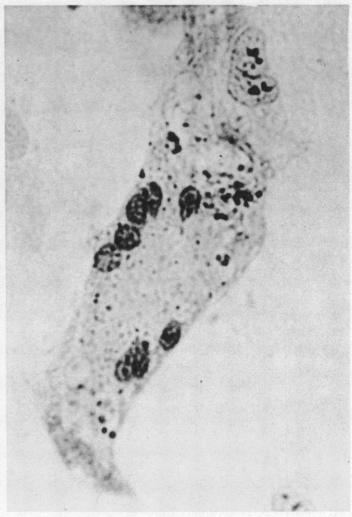

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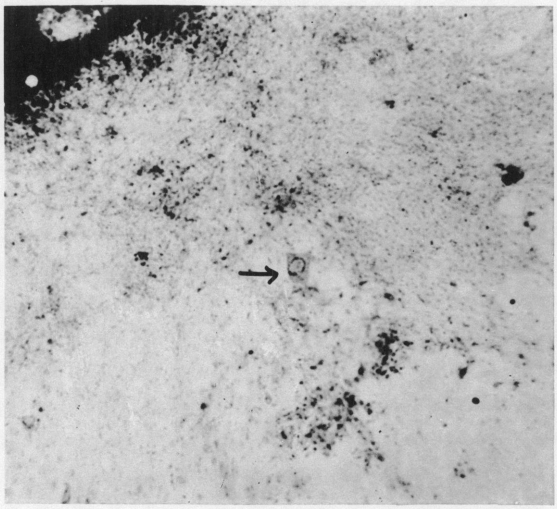

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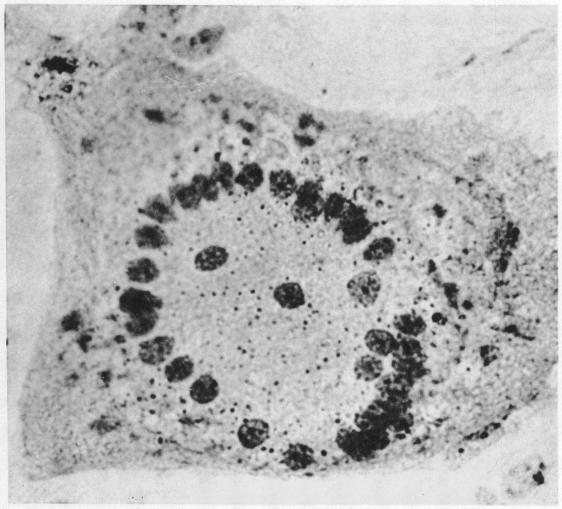

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PLATE 46

1. Six-day thymus culture, showing common binucleated epithelial cells. Harris' haematoxylin and eosin. ×540.

2–6. Fourteen-day thymus culture, showing multinucleation. Harris' haematoxylin and eosin. ×560. 2. Binucleated giant cell. No phagocytized material. 3. Multinucleate cell with five nuclei which seems to have been derived from the type of cell shown in 2. 4. Binucleated giant cell containing ingested pigment granules. 5. Multinucleated giant cell with eight nuclei, containing ingested pigment granules. Nuclei seem to be arranged about a central core. 6. Elongated multinucleated giant cell with eight nuclei, arranged peripherally. Note ingested pigmented granules.

7. Lower magnification of the two-week old thymus culture to show the location of a multinucleated giant cell containing 37 nuclei, within the area of proliferation. Harris' haematoxylin and eosin. ×34.

8. Higher magnification of cell shown in 7. Most of the 37 nuclei are arranged about a central core. Note ingested pigment granules as well as other phagocytized material. Harris' haematoxylin and eosin. ×560.

PLATE 47

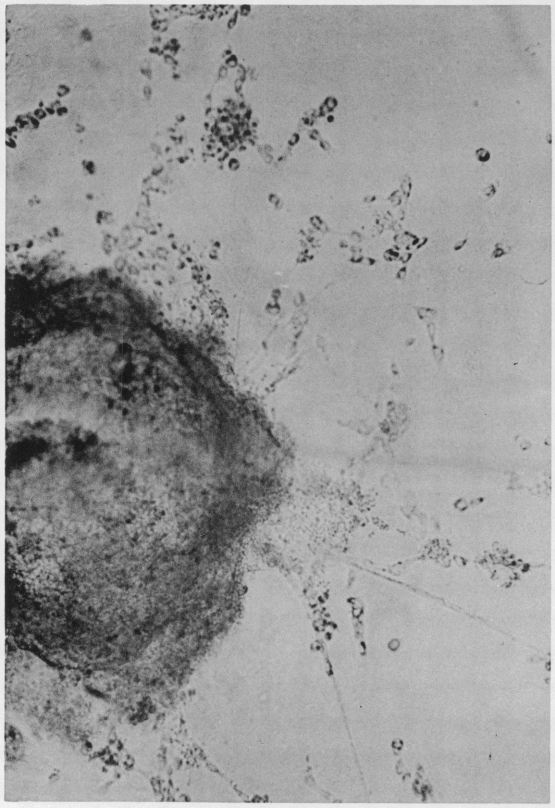
1. Three-day thymus culture, showing beginning of fibroblast proliferation at center. Living. ×100.

2. Higher magnification of living fibroblasts in a four-day thymus culture. Living. ×650.

3. Two-week-old thymus culture, showing a sheet of epithelium supported in part by intermingling fibroblasts. Harris' haematoxylin and eosin. ×560.

4. Three-day thymus culture stained with methyl green-pyronin. The nuclei of the normal thymocytes and epithelium stain green, whereas the cytoplasm and epithelial nucleoli are stained pink. Degenerating thymocytes which appear dark are stained deep purple; some of the ring forms have a light green center. The black intracellular granules are pigment. ×560.

5. Three-day thymus culture stained with methyl green-pyronin, after prior digestion with ribonuclease. The cytoplasm and nucleoli of the epithelial cells have taken no pyronin stain. The culture is predominantly green, with the exception of the light greenish purple of some of the degenerating thymocytes. ×560.



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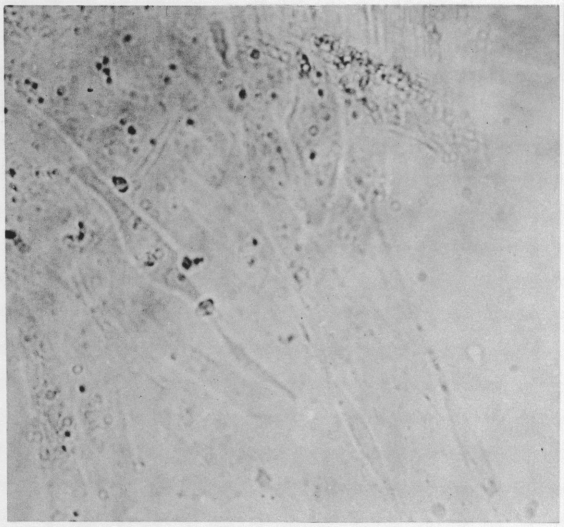

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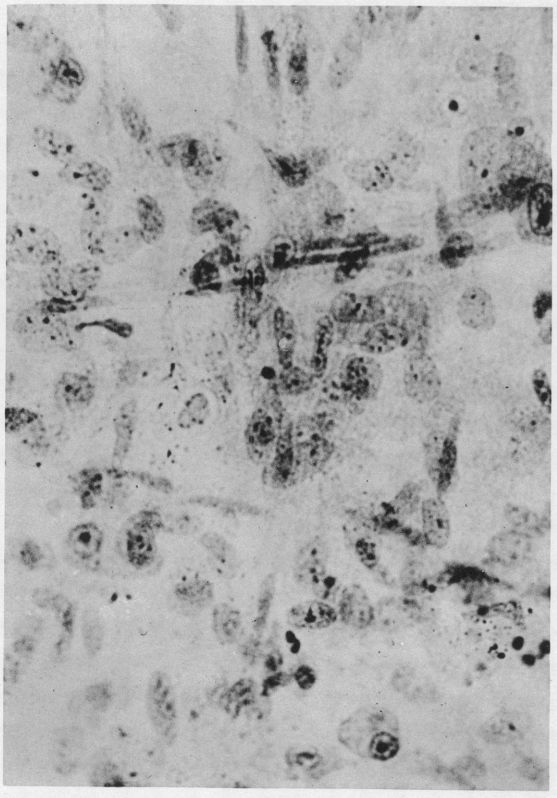

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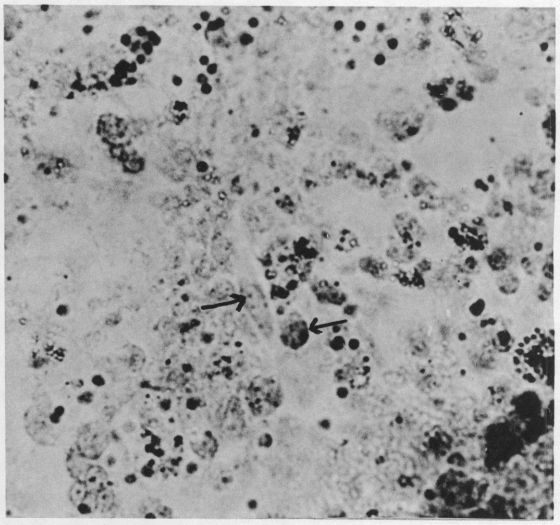

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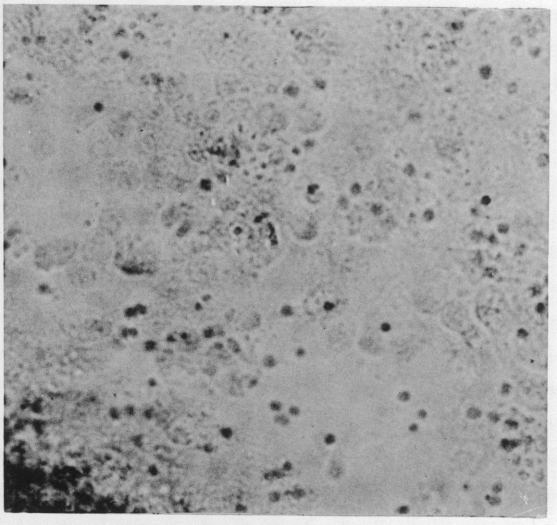

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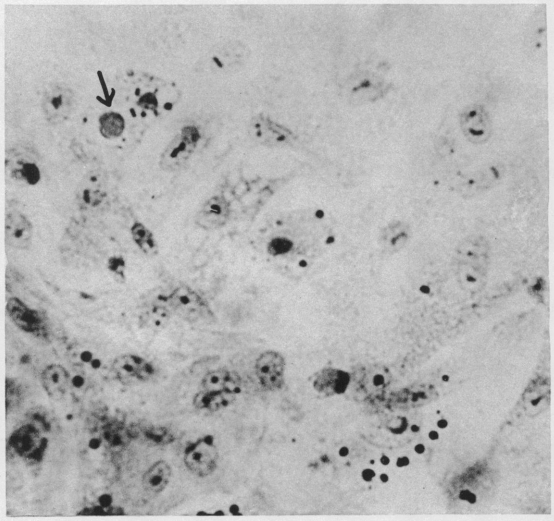

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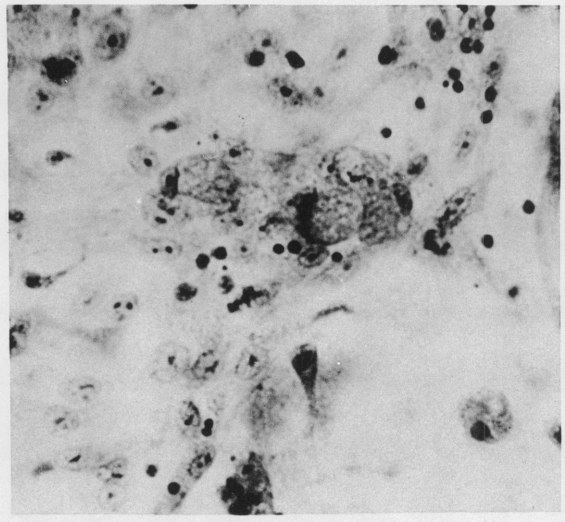

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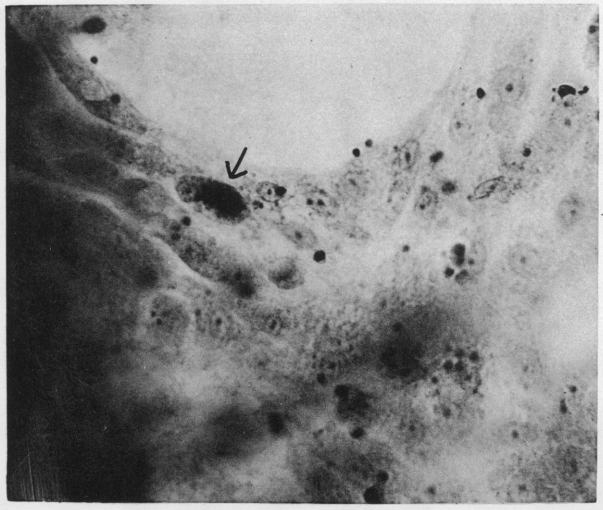

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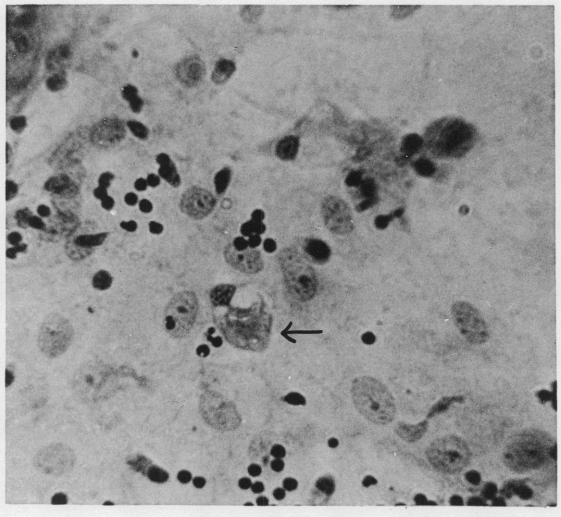

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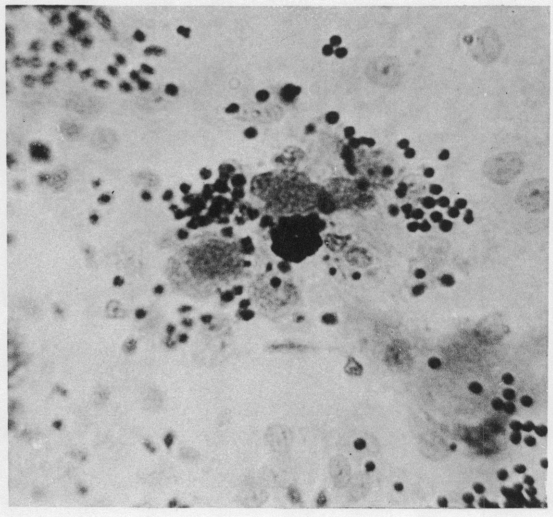

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PLATE 48

1. Epithelial sheet in a 10-day thymus culture, stained by the Bauer-Feulgen technique. There was no loss of reaction after prior digestion with saliva. Note single positive inclusion in the cytoplasm of the cell at upper left. Eccentric position of the nucleus is evident. ×560.

2. The same culture as is shown in 1, showing a group of cells in which the entire cytoplasmic area contained Bauer-Feulgen positive material. ×560.

3. Epithelial sheet in a four-day thymus culture stained by the Periodic Acid-Schiff method. Note PAS-positive material in a cell left of center at the growing edge. There was no loss of PAS-positive material after prior digestion with saliva. ×560.

4. Epithelial sheet in a three-day thymus culture, stained with Alcian blue, showing a single cell containing Alcian blue-stained inclusion. Note eccentric position of nucleus. ×560.

5. The same culture as is shown in 4, showing a group of cells in which the cytoplasm contained much Alcian blue-staining material. ×560.

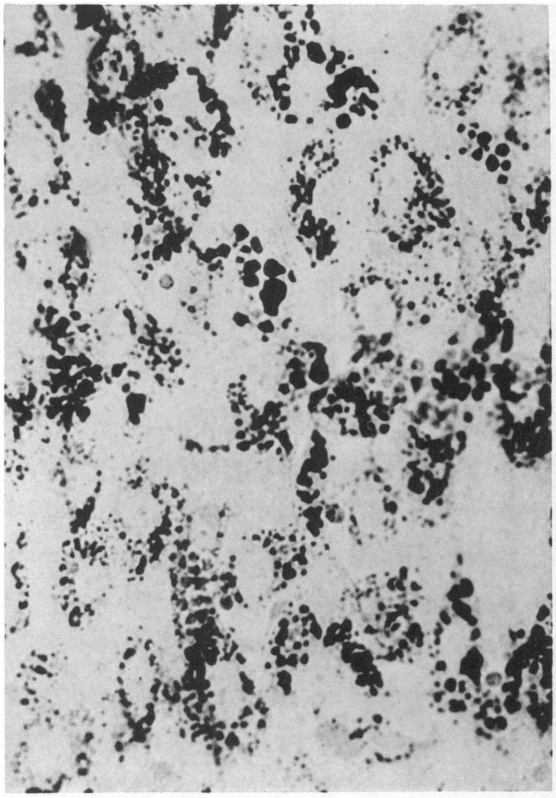
PLATE 49

1. Epithelial sheet in a two-day thymus culture stained with Sudan Black B. Note extranuclear distribution of lipid droplets of various sizes and shapes. ×560.

2. Epithelial sheet in a three-day thymus culture stained for neutral fats with Sudan IV. In addition to the droplets of irregular size and shape, note especially Sudan IV-positive rings and arcs. ×560.

3. Three-day thymus culture stained for phospholipids with Baker's Acid Hematein. The blue stain is localized in the cytoplasm of the epithelial cells as well as in degenerating thymocyte nuclei. The epithelial nucleoli are merely of a different density than the nuclei, but are not stained by the acid hematein. ×560.

4. Three-day thymus culture stained with Baker's Acid Hematein after pyridine extraction. Note the lack of stain in the cytoplasm of the epithelial cells but the persistence of the stain in the nucleoli and the degenerating thymocytes. ×560.



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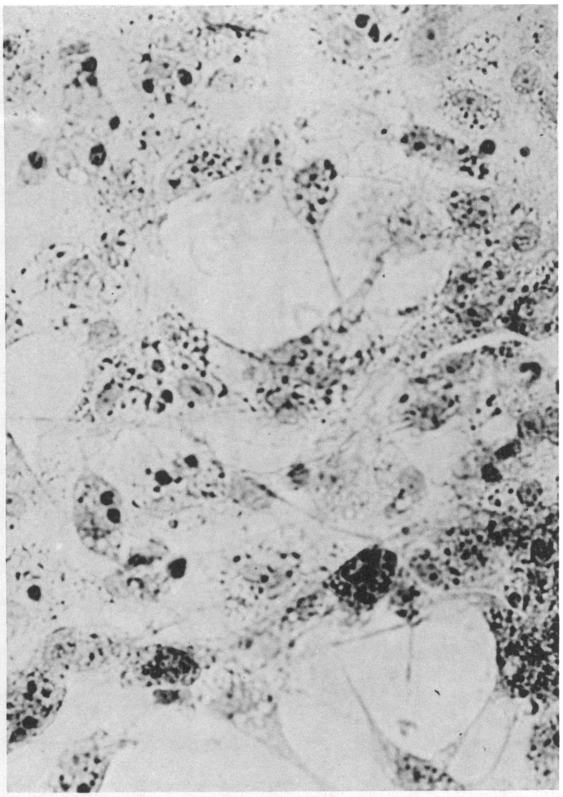

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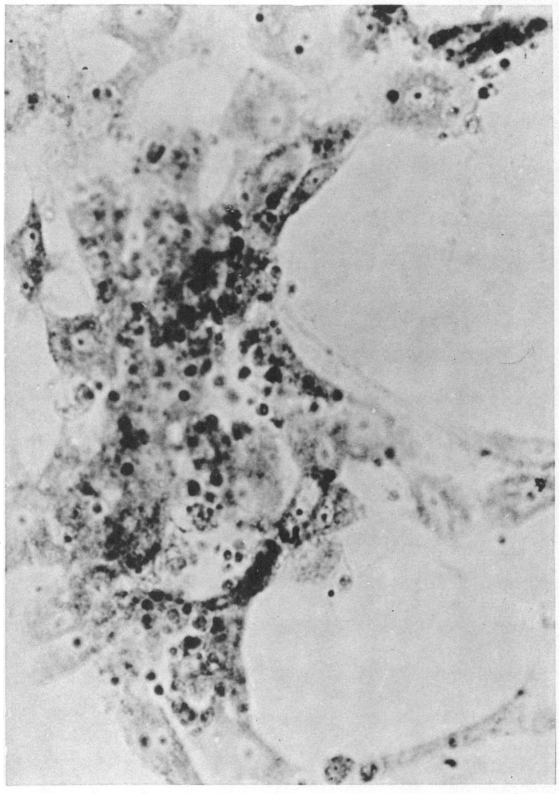

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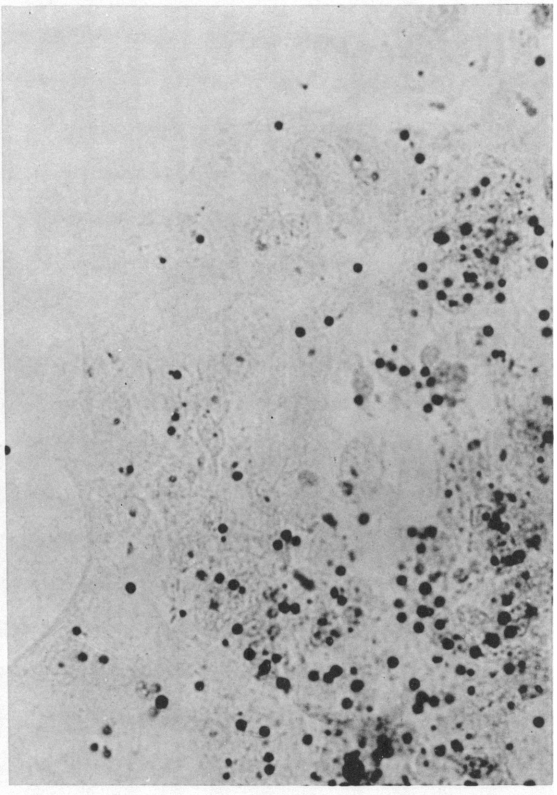

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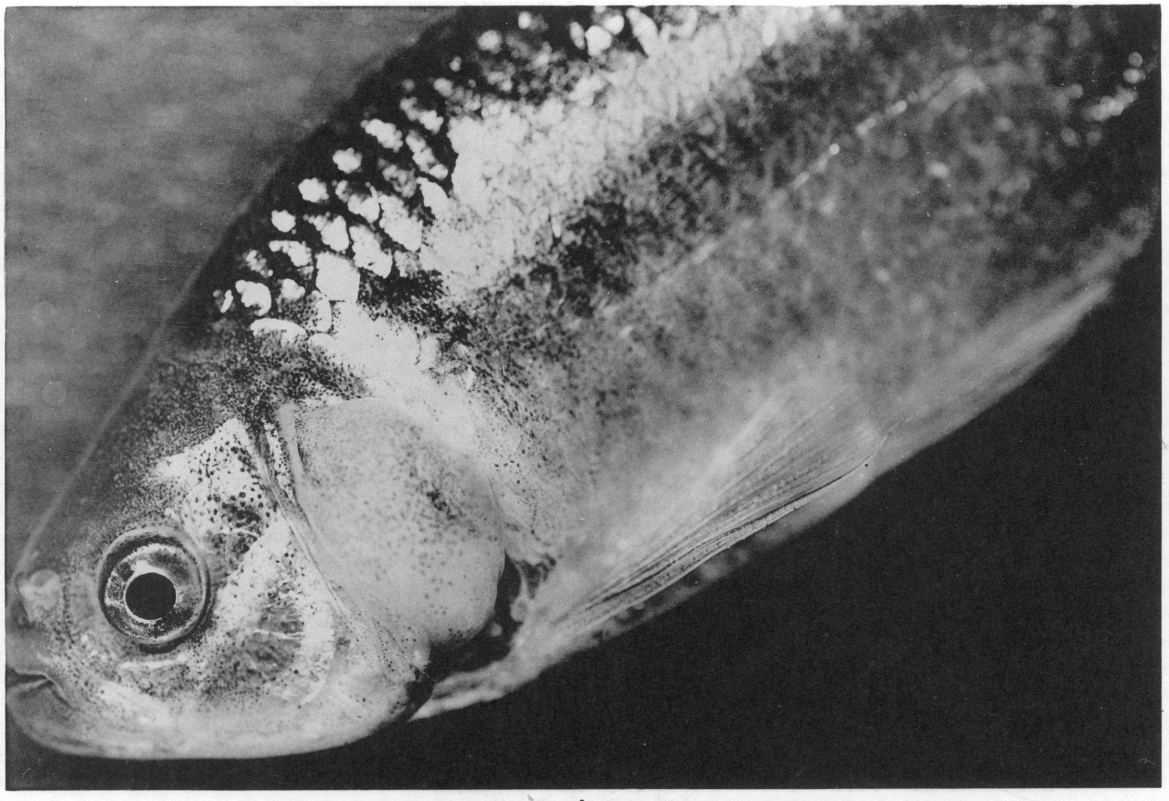

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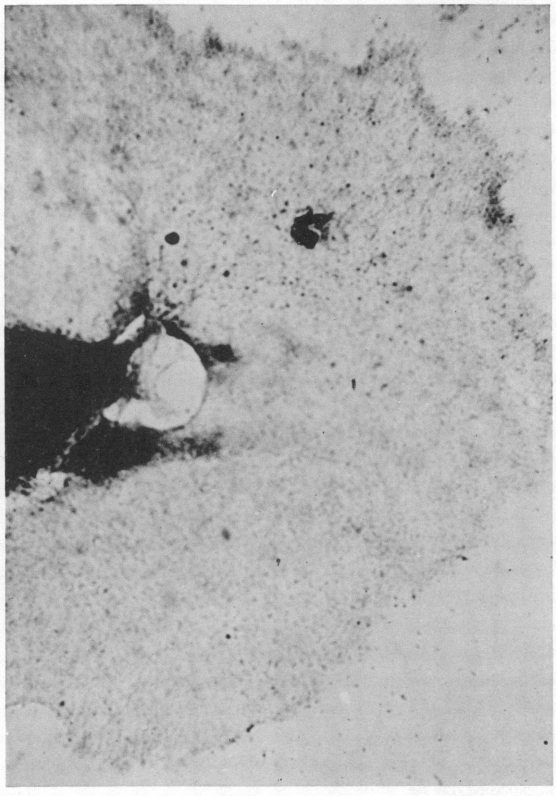

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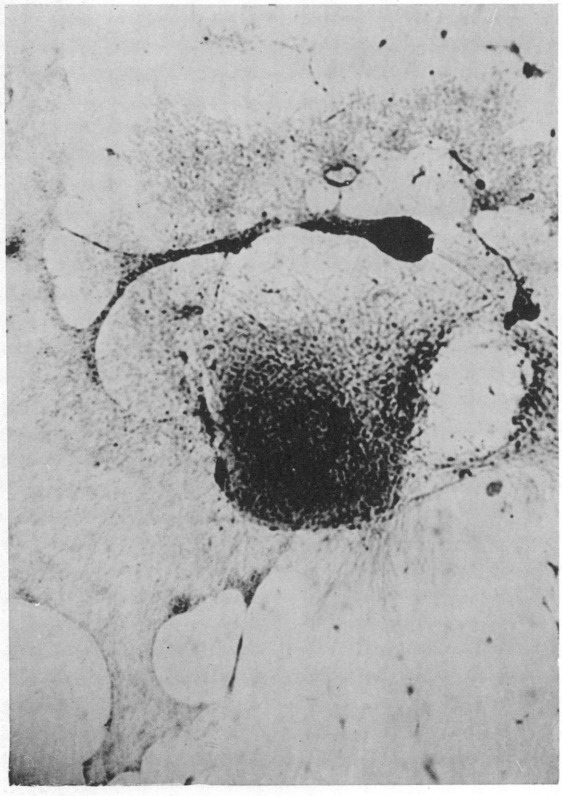

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PLATE 50

1. Photograph of the living fish, showing the tumor mass of lymphosarcoma (Tumor A) protruding from the branchial cavity. The operculum is vertical to the plane of the photograph as a result of an outward and forward deflection by the tumor mass. Courtesy of P. Rasquin and E. Hafter. ×4.

2. Three-day culture of lymphosarcoma (Tumor A), showing extensive proliferation of epithelium in the form of a regular-edged sheet. Harris' haematoxylin and eosin. ×43.

3. Three-day culture of lymphosarcoma (Tumor A), showing predominantly epithelial sheet proliferation with a subsequent proliferation of spindle cells. Harris' haematoxylin and eosin. ×43.

PLATE 51

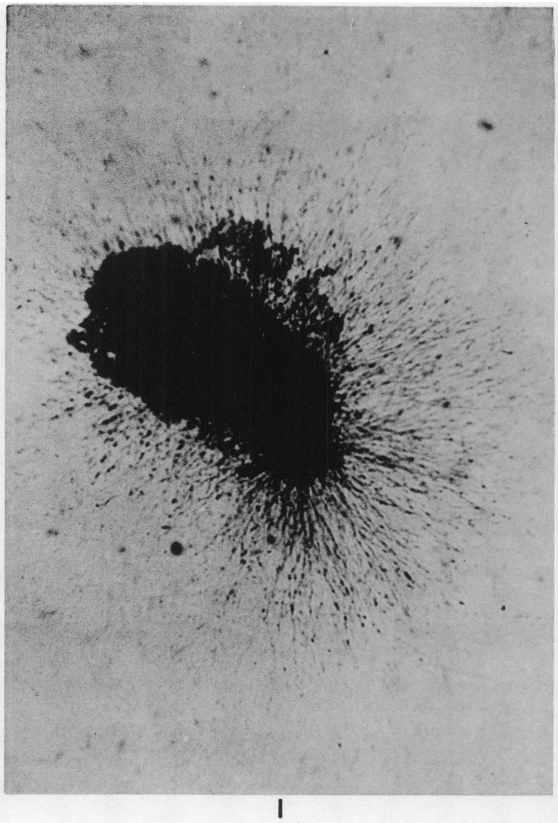
1. Three-day culture of lymphosarcoma (Tumor B), showing predominantly spindle-cell proliferation. Harris' haematoxylin and eosin. ×43.

2. Three-day culture of lymphosarcoma (Tumor B), showing spindle-cell growth (at growing edge, lower left) as well as some epithelial sheet proliferation (upper right). Harris' haematoxylin and eosin. ×43.

3. Three-day culture of lymphosarcoma (Tumor B), showing spindle-cell proliferation consisting of two types of spindle cells. Harris' haematoxylin and eosin. ×106.

4. Higher magnification of the culture shown in 3. Focus is on the spindle-like epithelium growing on the cover slip. Harris' haematoxylin and eosin. ×560.

5. Higher magnification of the culture shown in 3. Focus is on the true spindle cells growing deeper in the clot. Harris' haematoxylin and eosin. ×560.



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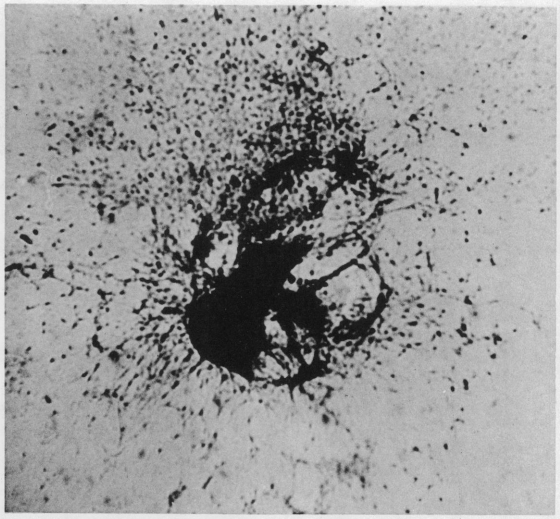

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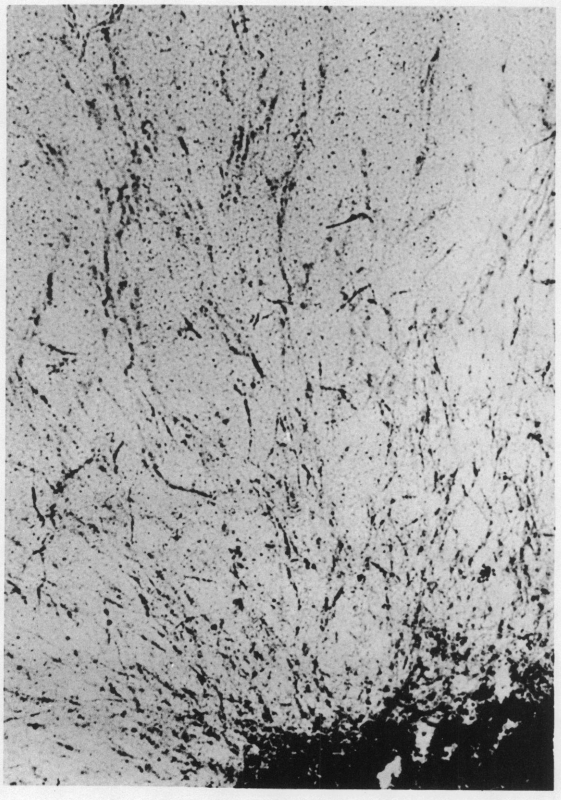

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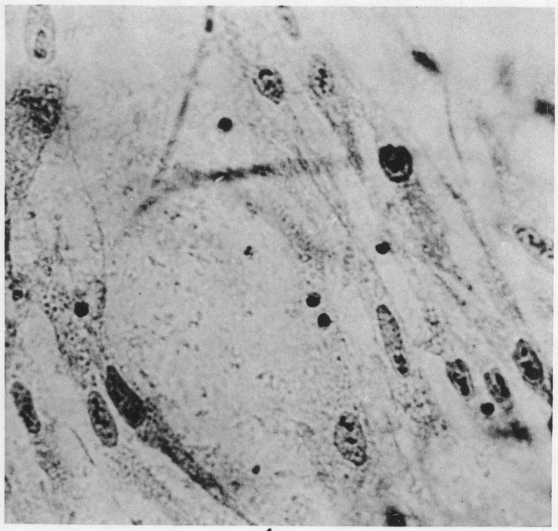

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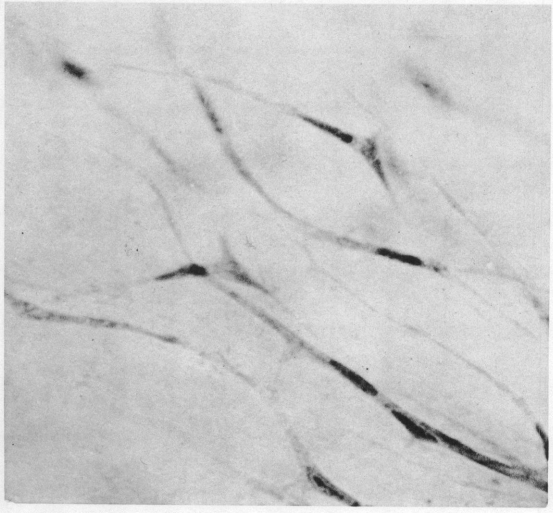

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TABLE 4

HISTOCHEMICAL DETERMINATIONS OF EPITHELIAL CYTOPLASMIC INCLUSIONS

Stain	Reaction
Glycogen and mucopolysaccharides	
Bauer-Feulgen	+
Bauer-Feulgen, after pretreatment with saliva	Fast
Periodic acid-Schiff (PAS)	+
PAS, after pretreatment with saliva	Fast
Best's carmine	−
Mayer's mucicarmine	+
Alcian blue	+
Lipids	
Sudan Black B	+
Sudan IV	+
Baker's Acid hematein	+
Baker's Acid hematein, after pyridine extraction	Labile

free phospholipids such as were found in the epithelial cytoplasmic granules. One of the reasons suggested by Cain (1947) that some substances do not stain with acid hematein but do stain after pyridine extraction lies in the difference in fixatives for both techniques. The former (acid hematein) involves the use of calcium chloride and formaldehyde, a non-precipitant type of fixative, while the latter (pyridine extraction followed by acid hematein) utilized Bouin's fluid, a precipitant fixative, by which the phospholipids were precipitated and thereby concentrated.

The histochemical reactions are summarized in table 4.

EFFECTS OF TREATMENT WITH ADRENOCORTICAL STEROIDS

There was no apparent inhibition of thymocyte migration regardless of the amount of adrenal hormone incorporated in the medium. The extent of migration appeared rather to be dependent on the nature of the fragment originally selected. Actively wandering tailed and ameboid cells were observed, in addition to the more stationary rounded cells. Healthy as well as degenerating cells were always present, just as they were in untreated normal thymus cultures. Mitotic figures, though generally infrequent, were observed in cultures treated with each of the concentrations of hormones, but none was seen in the controls. On the whole, the condition of the cells appeared to be correlated with the age of the culture rather than with the concentration of the hormones that were administered.

In those cultures made from fragments which were allowed to soak from one and one-half to three hours in the hormone solutions prior to being cultured, the condition of the thymocytes was also variable. There were many apparently normal cells among the large number of degenerated cells. In general, however, this method of hormone administration was too injurious to the tissue, which became so fragile that it fell apart on touch, and cultures of intact fragments were not feasible.

The measurements made of the diameters of thymocyte nuclei in cultures treated with cortisone were not statistically different from those of normal untreated thymocytes (table 2).

An objective count of normal and degenerating thymocytes in the tissue cultures was not possible because of the high percentage of degenerating cells always present and the irregular nature of the migration. Frequently, entire areas of normal healthy cells were seen side by side with large clumps of degenerating cells. However, cells were counted in a preliminary series of histological sections, made from whole thymuses allowed to soak in cortisone 24 hours prior to fixation. Table 5 shows the total number of cells counted and the percentage with pycnotic nuclei from the peripheral and more interior areas. The only real and conclusive effect observed was that the number of degenerating cells was much greater in the peripheral region, owing to better penetration of the hormone. Also, the Holtfreter controls showed the smallest percentage of cells with pycnotic nuclei, although the difference between these and some of the experimentals (especially the thymus subjected to the greatest concentration of cortisone, 200 μg./cc.) was frequently slight. On the basis of the few thymuses counted, there was no direct relationship between the number of destroyed cells and the concentrations of hormones used. Based on the fact that all the treated cultures showed a greater number

TABLE 5

PERCENTAGE OF PYCNOTIC THYMOCYTES IN THYMUSES EXPOSED TO HORMONE TREATMENT FOR APPROXIMATELY TWENTY-FOUR HOURS

Thymus No.	Treatment	Peripheral Region		Central Region	
		Total No. of Cells	Per Cent Pycnotic	Total No. of Cells	Per Cent Pycnotic
76	Holtfreter control	2027	81	2119	21
82	0.1 μg. cortisone	2139	91	2008	46
80	1.0 μg. cortisone	2015	88	2070	54
79	10.0 μg. cortisone	2001	94	2017	29
87	200.0 μg. cortisone	2050	83	2005	29

of cells with pycnotic nuclei than did the controls, perhaps a slight effect is indicated. In order to establish this effect more concretely with this method of experimentation, many more glands must be used and probably more than 2000 cells for each gland must be counted. Such an experiment was not attempted, however, in view of the fact that the drastic nature of this method of exposure to the hormone introduced a second variable. No doubt there was an additive effect of the hormones and unfavorable milieu. Furthermore, such an approach did not really answer our purpose, which was to determine the direct effects of the hormones on normal, healthy-growing thymocytes *in vitro*.

Although in general the cultures were not allowed to grow long enough for epithelium or fibroblast proliferation prior to treatment with the hormones, several cultures occasionally showed such cells. No deleterious effects were observed in either the epithelial cells or the fibroblasts, and mitotic divisions were not interrupted.

LYMPHOSARCOMA

The external appearance of the two tumors was similar. In both, a grayish white, opaque mass protruded from the left gill cavity, pushing the operculum out at almost right angles to the body of the fish (pl. 50, fig. 1). A thin, membranous epithelium containing scattered melanophores surrounded the tissue. At the time of biopsy, the tumor felt soft but resilient.

A histological description of one of the tumors, before and after hormone therapy, was presented by Rasquin and Hafter (1951). The tissue came from the same lymphosarcomatous fish from which biopsy material was taken for our *in-vitro* studies. The tissue, taken at biopsy before treatment, was composed of small, closely packed lymphocytes supported by a fine, reticular stroma. The second lymphosarcoma presented a similar histological picture (Rasquin and Rosenbloom, 1954).

IN-VITRO MORPHOLOGY OF TUMOR TISSUES

Fragments of the tumors grown in tissue culture resembled fragments of normal thymus tissue under the same conditions. Within the first 24 hours *in vitro*, there was a mass migration of lymphocytes, frequently in a halo-like formation. The majority of the cells appeared in the rounded state, although tailed and ameboid cells were also seen. Measurements of the diameters of the lymphocyte nuclei revealed that these cells were larger than thymocytes of normal thymus tissue *in vitro*. Table 2 presents some of these measurements and gives the statistical significance of the differences. Various stages of mitosis in the lymphocytes were observed in 29 per cent of the cultures of Tumor B, and in none of the cultures of Tumor A. Occasionally amitotic divisions were seen in cultures of both tumors that resembled the amitosis in normal thymus tissue. In addition, all degrees of degeneration were observed, ranging from slight pycnosis of the nucleus to complete fragmentation of the cell.

Epithelial cells and fibroblasts also made their appearances within the first 24 hours of *in-vitro* growth. It is interesting to note that, although the outward appearance of both tumors was similar, the growth patterns in tissue culture, exclusive of the lymphocytes,

were markedly different. In Tumor A vast sheets of epithelial cells were predominant, whereas in Tumor B a radial proliferation of fibroblasts was most in evidence. Both epithelial cells and fibroblasts, however, were present to some degree in almost all the cultures.

The epithelial sheets in Tumor A were one cell layer to several cell layers thick, with a smooth growing edge (pl. 50, fig. 2). Holes in the sheet were common. The cells were large, flat, and polygonal and usually contained many cytoplasmic vacuoles. Eosinophilic inclusions, basophilic nuclear fragments, and melanin granules were also occasionally present in the cytoplasm of these cells, which suggests some phagocytic activity. The size and shape of the nuclei and nucleoli varied considerably, and occasionally binucleated and multinucleated cells were seen. Mitotic divisions were observed in only one culture. In addition to the sheet formation, the epithelial cells sometimes appeared as individuals, in small groups or as strands. These cells were spindle-shaped, and anastomoses were frequent. Some growth of fibroblasts occurred in most of these cultures, although not extensively (pl. 50, fig. 3). Proliferation was generally radial, sometimes tangential, and frequently the cells were interspersed throughout the epithelial sheet. Mitoses appeared in only 10 per cent of the cultures.

In contrast, proliferation of fibroblasts in Tumor B was extensive, and mitotic figures occurred in large numbers in almost every culture (pl. 51, fig. 1). Some cultures showed epithelial sheet growth as well (pl. 51, fig. 2). There appeared to be two types of fibroblasts; the typical spindle cell, with its long, oval nucleus surrounded by little cytoplasm, and the very long, fine, almost filamentous cell with branched processes (pl. 51, figs. 3–5). In general, the growth was radial and tangential, with considerable intermeshing of the cells. Usually a thin, single-layered sheet of epithelium was observed underlying this meshwork of fibroblasts. At the growing edge, the cells were extremely spindle-like and hardly distinguishable from the true fibroblast, except for the nucleus which was still typically epithelial. Occasionally, these spindle-like cells were also scattered throughout the meshwork of fibroblasts. Mitotic figures in the fibroblasts appeared in approximately 50 per cent of the cultures.

Macrophages were observed throughout the cultures of both tumors and were frequently heavily laden with lymphocytic debris and melanin granules or clumps, presumably from disintegrated melanophores.

A comparison of the two tumors with normal thymus tissue *in vitro* indicated that Tumor A resembled normal thymus in its growth patterns *in vitro* more closely than did Tumor B. In addition, Tumor B was more proliferative, a fact evidenced by extensive growth and a high rate of mitosis. The latter tumor also contained more fibrous tissue, as demonstrated by the extensive fibroblastic proliferation.

Effects of Treatment with Adrenocortical Steroids and Adrenotropic Hormone

The extent of migration or proliferation of cells subjected to the various treatments could not be considered a criterion of effect, because all cultures were first allowed to grow undisturbed in a routine medium for 48 hours. Therefore an effect was sought in the condition of the individual cells as well as in the rate of mitosis for a given cell type.

There was no consistent pattern among the treated or untreated cultures. In most cases, presumably normal cells coexisted with degenerating cells. Occasionally, some of the cultures that had received hormone therapy presented a far healthier picture than control cultures.

On the whole, Tumor A showed very few dividing cells. There were no dividing lymphocytes in either experimental or control cultures. The only epithelial cells that exhibited occasional mitoses were found in 50 per cent of the cultures treated with compound A. Some dividing fibroblasts were also observed in cultures subjected to compound A, as well as in the untreated controls.

In Tumor B the rate of mitosis was generally high, especially as regards the fibroblasts. Under both sets of experimental conditions, in which the hormones were included in the wash or directly in the culture medium, all the cultures, with the exception of those treated with compound E, showed large numbers of dividing fibroblasts. No dividing

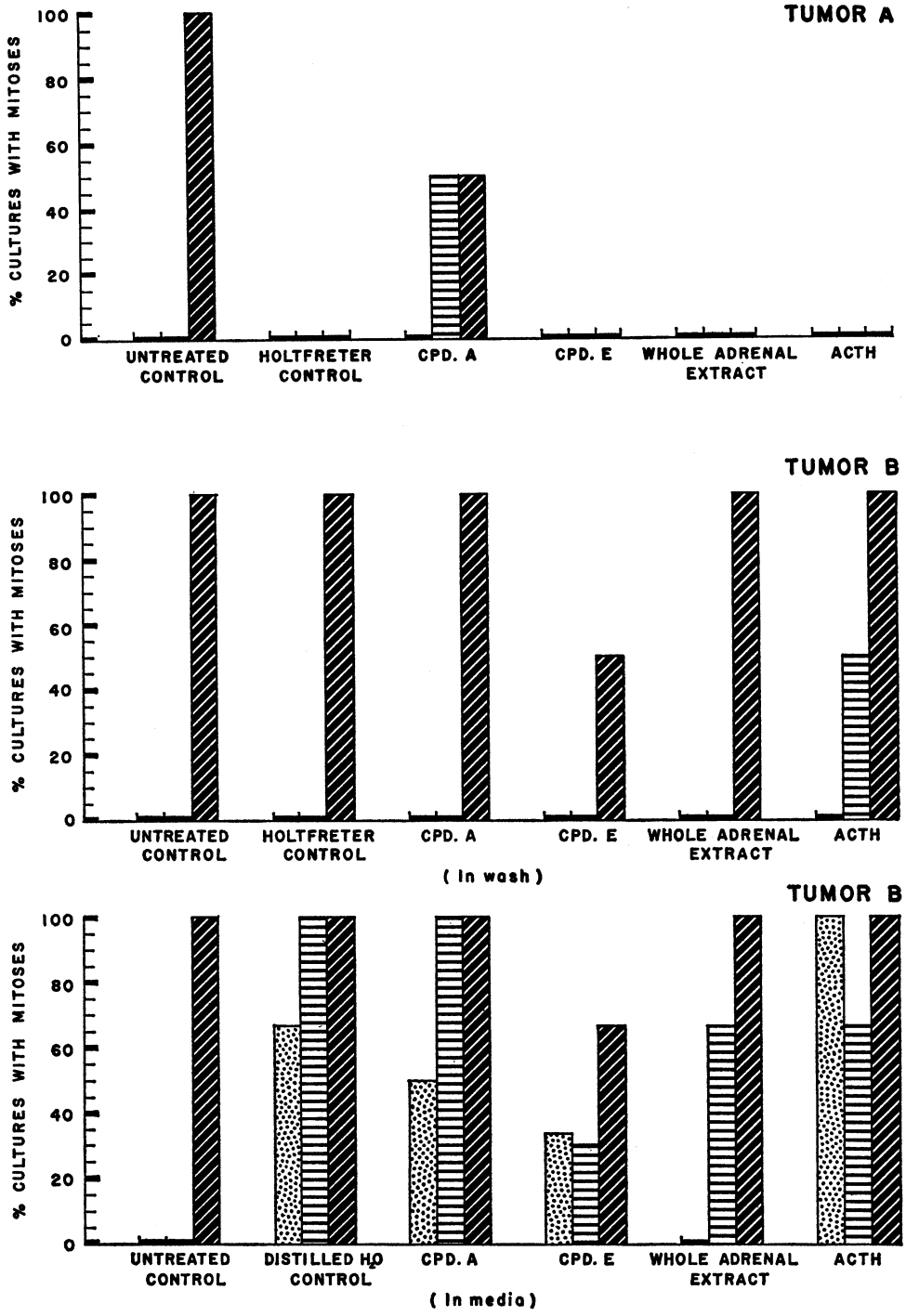

Fig. 1. Percent of cultures showing mitotic divisions in lymphocytes, epithelial cells, and fibroblasts in Tumors A and B, under different conditions. Lymphocytes are represented in the first column by stippling. Epithelial cells are represented by horizontal stripes in the second column. Fibroblasts are represented by diagonal stripes in the last column.

lymphocytes or epithelial cells were present in the untreated controls. In addition, there were no dividing lymphocytes in any of the cultures washed in the various substances, but 50 per cent of those cultures subjected to an ACTH wash showed mitoses in epithelial cells. In cases in which the substances were included directly in the medium, the results were somewhat different. Some dividing lymphocytes were seen in cultures under all conditions, with the exception of those treated with whole adrenal extract. Almost all the cultures showed some divisions in epithelial cells, except for 70 per cent of the cultures subjected to compound E. The percentage of cultures showing mitotic figures under experimental and control conditions, is presented graphically in text figure 1.

A comparison of the measurements of lymphocyte nuclear diameters in untreated and compound E-treated cultures failed to show any statistically significant differences. The values for these determinations can be found in table 2.

DISCUSSION

THE HISTOLOGY of the normal thymus of the teleost *Astyanax mexicanus* has been described by Hafter (1952) in a study of age involution. In general, those structures observed in histological sections were also observed in tissue culture. However, owing to the nature of "unrestricted" growth *in vitro*, some of the cell types that ordinarily occur in small numbers and are quite obscured by the dense parenchyma *in vivo*, are given new impetus to migrate and proliferate, which would account for the abundance of epithelial reticulum seen in our cultures and not in tissue sections. Hafter reported that none of these reticular cells is phagocytic. *In vitro*, however, fragmented as well as entire thymocyte nuclei were sometimes seen in the cytoplasm of these cells, which is in accord with the *in-vitro* studies of Pappenheimer (1913) and Wassen (1915) on frog thymus, Murray (1947) on rabbit thymus, and Trowell (1949b) on rat thymus. All these authors reported the presence of thymocytes in epithelial cells. In a control experiment, in which chick fibroblasts (ordinarily not considered phagocytic) and thymus were grown side by side in the same culture, Trowell also observed thymocytes in the fibroblasts. Perhaps, then, the mechanism by which the thymocytes enter the epithelial cells is not phagocytosis. Popoff (1927) concluded that these cells were not phagocytic, because they did not store lithium carmine. Likewise, in our cultures containing trypan blue, none of the epithelial reticular cells was observed to pick up the dye. The only phagocytic cells observed by Hafter in *Astyanax* thymus *in vivo* were occasional macrophages, which were few in number, regardless of the age of the animal. In contrast, our *in-vitro* experiments indicate considerable numbers of macrophages, either partially or completely laden with ingested material. These cells have also been observed in thymuses *in vitro* of other animals by Tschassownikow (1927), Popoff (1927), Emmart (1936), and Murray (1947). It is highly probable that the macrophages did not migrate out as such from the original explant, but developed as transformations *in vitro*, perhaps as Tschassownikow (1927) and Popoff (1927) have shown, from the local thymocytes. Bloom (1928) has also shown that typical polyblasts or macrophages arose by "individual differentiation of the explanted thymocytes." Trowell (1958), in a study of cultures of rat lymph nodes, introduced the concept of a lymphocyte⇋macrophage cycle. All stages of transition were also observed in our cultures, from wandering thymocytes to what we have referred to, on the basis of activity and stainability, as "eosinophilic wandering cells." Hafter (1952) reported similar cells in the thymus *in vivo* which, however, were probably in a different stage of development. Because of the many transitional stages, these cells have been given various terms by different authors, although their descriptions all seem to point to the same cell derived from the thymocyte or lymphocyte. Catton (1951), in a study of blood cell formation in certain teleost fishes, referred to them as "coarse granulocytes." In cultures of lymphoid tissues, they have been referred to as "eosinophiles" (Emmart, 1936), "myelocytes" (Bloom, 1937), and "early myelocytes" (Murray, 1947). Bloom defined the myelocyte as a cell with a lymphocyte-like nucleus and eosinophilic granules. Earlier, Jordan and Speidel (1924), reported the formation of eosinophiles from lymphocytes, with transition stages between the two, in teleost spleen.

It is generally believed that structures that can compare with true Hassall's corpuscles are rarely found in the teleost thymus. However, according to Salkind (1912) the reticulum cells, which have a decreased secretory activity, in the thymus of older fish become modified either into Hassall's corpuscles, giant cells, cysts, or pseudomyoid cells. In *Astyanax*, Hafter (1952) reported that the only structures that were at all similar to Hassall's corpuscles were single, hypertrophied, reticular cells, or two or more of these cells grouped together. In tissue culture, we have observed the presence of "pearls" in the epithelial reticulum, which were composed of concentrically arranged cells, occasionally with a central core of debris. Popoff (1927) also reported the presence of scattered "pearls" in his cultures of rabbit thymus.

Tschassownikow (1927) considered that one of the ways in which epithelial islands were formed in cultures of rabbit thymus was through the formation of "pearls" in the compact epithelial layer. The morphological similarity between these structures and Hassall's corpuscles in higher vertebrates is striking. While Tschassownikow felt the "pearls" bore some resemblance to early developmental phases of Hassall's corpuscles, he did not see any real Hassall's corpuscles in his cultures because degeneration set in too soon.

It is interesting to note that the presence of epithelial "pearls" is quite diagnostic of some epidermoid malignancies, for example, squamous cell carcinoma (Allen, 1954). In both instances, namely, teleost thymus tissue *in vitro* and the epidermoid carcinoma, the common factor is rapidly proliferating epithelium. Perhaps, then, the presence of "pearls" in our cultures is to be associated not with Hassall's corpuscles, but rather with the rapidly proliferating epithelial tissue as such, a possibility that is certainly thought-provoking, when one considers the similarity of the situation that exists in tissue cultures and malignant growths. In the former the constricting and confining influences of the intact organism no longer prevail, while in the malignancies the normal reins or checks are disturbed and uncontrolled growth results.

Much attention has been focused on the origin, method of formation, and significance of giant cells since the classic description by Langhans in 1868 of the giant cells in tuberculosis. These cells also normally occur in many of the lower vertebrates, including fish (Salkind, 1912; Baitsell, 1924; M. R. Lewis and W. H. Lewis, 1926). Giant cells have been observed in a variety of tissues, both normal and pathologic, especially those of a lymphoid nature. In particular, several investigators have reported their presence in the thymus gland (Salkind, 1912; Popoff, 1927; Emmart, 1936).

There is much confusion in the literature as to the precise mode of origin of giant cells, as well as the stimulus causing their formation. According to Salkind (1912), epithelial cells of the thymus have a tendency toward engulfment and intracellular and extracellular digestion. The giant cell is merely one aspect of the cell during the digestive process. With the tissue-culture technique, direct observations were made on spontaneously occurring as well as experimentally produced giant cells, which yielded several theories as to their formation. Among those who claim that a fusion of cells is the method of multinucleated giant cell formation are Lambert (1912), Maximow (1924), Popoff (1927), Pires Soares (1937), and Goldstein (1954a, 1954b). A second theory, that of formation by repeated nuclear divisions without cytoplasmic divisions, has also received considerable support by Weil (1913), Macklin (1916), Lewis and Webster (1921), M. R. Lewis and W. H. Lewis (1925, 1926), W. H. Lewis and M. R. Lewis (1925), and W. H. Lewis (1925–1926; 1926). Several investigators, such as Jordan (1925), Barta (1926a, 1926b), and Forkner (1930), recognize the existence of both fusion and amitosis as methods of giant cell formation. Weiss and Fawcett (1953) claim that the majority of giant cells seem to be formed by the coalescence of individual cells. However, the shape of some of the nuclei was also suggestive of amitotic division. Another explanation of the formation of these cells is that presented by Ackerman, Knouff, and Hoster (1952), in a study of human lymph nodes in tissue culture. The authors refer to the foreign body giant cell as a phagocytic clasmatocyte which has engulfed nuclear fragments as well as entire cells such as lymphocytes.

While we have not followed the formation of giant cells in our living cultures, the fixed and stained specimens reveal the presence of two types of these cells. Only in the Langhans type of cell were there any indications as to the possible mode of formation of the multinucleated condition, and here our conclusions agree with those of Lewis and co-workers. Our strongest evidence comes from the shape of some of the nuclei. The fact that many of the nuclei in the ring are attenuated toward the center suggests recent amitotic divisions. In some instances, one can almost match up the two daughter nuclei diagonally across from each other. No evidence of fusion has been observed, but it is conceivable that such may have been a method of formation of some of the giant cells. The unusual regular arrangement of the nuclei in the Langhans type of cell would then be accounted for by a

coalescence of cells, all of one type, about some foreign substance.

Just as there are various opinions among investigators as to the mode of formation of these cells, the reasons given for their formation are as varied and as speculative. Many workers believe that these cells are foreign-body reactions, since they can be produced experimentally with various stimuli (Timofejewsky and Benewolenskaja, 1927; Lambert, 1912; Weil, 1913; and Goldstein, 1954a). Lewis and Webster (1921) attributed the formation of giant cells to the excess of waste products accumulated in the cell from ingested lymphocyte nuclei, which acts as a stimulus for amitotic nuclear divisions and thereby provides a means of utilization of the overabundance of ingested material. Barta (1926a) offered deficient oxidation as an explanation. By placing fragments of lymph node in different depths of a plasma clot, he claimed to have produced multinucleated cells in the deeper regions of the clot only, where there is the least amount of oxygen. Pires Soares (1937) disputed Barta's conclusions when he reported that giant cells also occurred at the periphery of the culture medium in which the oxygen supply was very good. It is unlikely that the giant cells in our cultures are the result of insufficient oxygen, as our clots were generally thin and quite uniform in depth throughout. In addition, most of the giant cells that we observed were in the more peripheral region of the zone of proliferation, where there were fewer cells per area. We are therefore more inclined toward the suggestion of Rottino and Hollender (1949b), who believe that the giant cells arise in response to some metabolite originating in the tissue when it is grown *in vitro*. Their conclusions are based on tissue culture studies of lymph nodes involved in Hodgkin's disease. *In-vitro* metabolism was emphasized, since the multinucleated giant cells were only rarely found in tissue sections of surgically removed nodes with Hodgkin's disease. It is interesting to note that only slight mention is made of giant cells in those studies of the thymus concerned only with histological sections, and in the description of the thymus of *Astyanax* by Hafter (1952) there is no mention of these cells. According to Weiss and Fawcett (1953), while the precise environmental conditions that determine the coalescence of epithelioid cells to form giant cells are obscure, cell crowding and low pH seem to favor their development. No determinations were made of the pH of our cultures, but multinucleated cells were observed in highly proliferative cultures in which there probably was crowding of the cells.

From the evidence presented, it seems highly probable that more than one type of giant cell exists, and that they may be formed by one or more methods, as the result of any number of stimuli. Obviously many questions concerning the giant cell remain to be answered, especially when we consider that most of the data presented are based on tissue culture studies. The situation *in vivo* is not yet understood.

In attempting to understand the nature of the cytoplasmic inclusions in the teleost thymus epithelium *in vitro*, we subjected our cultures to a series of histochemical determinations, the results of which indicate that these inclusions are not necessarily chemically homogeneous. Some cells had one or more granules that were Bauer-Feulgen positive as well as PAS positive, even after pretreatment with saliva, which suggests the presence of some polysaccharide other than glycogen. This was confirmed by a lack of stainability with Best's carmine. The fact that these inclusions stained with Mayer's mucicarmine and Alcian blue denotes the presence of an acid mucopolysaccharide, probably mucus. Hafter (1952) also observed the presence of mucus in some cells of the thymic reticulum in *Astyanax* and reported that similar cells have been described for other fishes. Smith and Thomas (1950) found glycogen in about 40 per cent of the small cortical lymphocytes of thymuses of young mice and kittens shortly after birth. Between 14 and 18 days, there was an abrupt drop which the authors explained as perhaps correlated with a marked change in food habits and growth at about that time. None of our cultures showed the presence of glycogen, which may be accounted for by the age of the fish which were approximately two months old. Such an age is considerably beyond the equivalent fourteenth day of development discussed by Smith and Thomas.

The majority of the epithelial cells possessed a preponderance of lipid inclusions which gave a heavy positive reaction with

Sudan Black B. That many of these fat droplets were phospholipid in nature was substantiated by a failure to stain with Baker's Acid Hematein after extraction with pyridine. Some of these droplets were either partially or completely surrounded by a neutral fat, as indicated by the presence of Sudan IV-staining rings or arcs. It is highly probable, therefore, that the majority of these cytoplasmic inclusions are of a complex chemical nature rather than simple chemical entities. The lipid-laden cells described by Loewenthal and Smith (1952) in the involuting mouse thymus are not comparable to the epithelial reticulum of our cultures. Such foamy cells as described by these authors were not observed in the fish thymus *in vitro*, which was not surprising, as we were not dealing with an involuting gland. In a study of the cytochemistry and morphology of human lymph node cells grown *in vitro*, Ackerman, Knouff, and Hoster (1952) reported a difference in the reactions *in vitro* from those in tissue sections. For example, the reticular clasmatocytes show more neutral fat and sudanophilic droplets *in vitro* than in tissue sections, which suggests that there may be an impaired fat metabolism *in vitro*. The authors also believed that certain cytochemical modifications take place with degeneration, one of the most obvious being an accumulation of lipid droplets. Weiss and Fawcett (1953) demonstrated the lack of stainable lipid in chicken monocytes. However, as they became transformed into macrophages in tissue cultures, fat droplets rapidly appeared. Again, an increase in fat droplets was observed in cultures when the cells were in poor condition. The large quantity of lipid material in our cultures was not the result of degeneration, as some of the determinations were performed on healthy, active, 48-hour cultures. Weiss and Fawcett also demonstrated the presence of phospholipids in some of the epithelioid cells. The variation in staining from cell to cell, they stated, could not be correlated with degeneration. It may reflect a real difference in the intracellular distribution of phospholipid, or it may merely represent one of the vagaries of this staining method.

Before any conclusions can be drawn as to the possible significance of the various histochemical reactions of the fish thymus cells in tissue culture, it is necessary to determine these reactions on tissue sections of the gland.

The well-known influence of the adrenocortical hormones on mammalian lymphoid tissue (Dougherty and White, 1945; Selye, 1946) was experimentally demonstrated in the lymphoid system of fishes, on *Astyanax*, by Rasquin (1951), Rasquin and Atz (1952), and Rasquin and Rosenbloom (1954).

Considerable effort has been made in an attempt to understand the mechanism by which the various hormones act on lymphoid tissue. In order to determine whether these effects are direct, acting on the lymphocyte itself as the immediate target, or indirect, reaching the lymphoid elements via the interrelationships of the intact organism, many investigators turned to the tissue culture technique (Heilman, 1945; Schrek, 1949; Krippaehne and Osgood, 1955; Vernon, 1958). The results are quite contradictory.

The fact that there is so much discrepancy among the reports of lymphocytes *in vitro* led Trowell (1953) to suggest that certain technical difficulties are inherently involved. With the methods employed, it has been virtually impossible to maintain a population *in vitro* of all living healthy lymphocytes for even a few hours. From the very start one always finds some dead cells, the number of which steadily increases during the culture period. This condition has also been noted in our cultures of fish tissue. At no time were the cultures devoid of degenerating forms. Frequently, groups of seemingly healthy cells were observed immediately adjacent to, or intermingled with, dead or dying cells. For this reason we reported that a count of normal versus degenerate cells was inaccurate as a valid criterion for determining the effects of various hormones on the thymocytes *in vitro*.

In a comparative study of cultures of fragments, cell suspensions, and whole glands, Trowell (1949a) found that the number of dead or degenerating cells was considerably lessened when whole lymph glands were cultured. Notably different in the methods of culture is the fact that the tissue used for fragments or cell suspensions must obviously undergo considerable trauma when being cut into many small pieces. Probably such damage to the cells is responsible in part for their early death.

In the present experiments, when intact thymuses were used, differences such as those reported by Trowell were not observed.

Under these circumstances, we found very poor growth or migration from the fragment unless the capsule had been cut.

Another explanation offered by Trowell (1952), and one that seems very probable, concerns the cytology of the lymphocyte itself, a cell with relatively much less cytoplasm than any other somatic cell. This paucity of cytoplasm is especially evident in histochemical demonstrations of nucleic acids in our cultures. The cells, for the most part, are composed of desoxyribonucleic acid in the nucleus, surrounded by a very thin ring of ribonucleic acid in the cytoplasm. It has been known that lymphocytes *in vivo* have a high rate of turnover of both proteins and nucleic acids, requiring therefore a continual synthesis of protein and nucleic acids for their maintenance (Andreason and Ottesen, 1945). When the ratio of nucleoplasm to cytoplasm is considered, it is extremely plausible that these cells do not possess the proper enzymic components for independent existence and must rely in part on other cells. It is highly probable that this condition was a significant contributor to the early death *in vitro* of single thymocytes in our cultures. In the intact gland, of course, the thymocytes maintain a close anatomical relationship with the reticular cells, which may explain the results of Emmart (1936) who states, "Thymocytes never disappear entirely as long as any epithelioid tissue remains visible in the cultures." Perhaps this can account for the persistence of the several healthy thymocytes in and about the sheet of epithelium in some of our older cultures.

Taking all these facts into consideration, Trowell (1952) devised a method of short-term cultivation for intact lymph nodes, using organ culture as contrasted to tissue culture.

Having established a method of maintaining lymphocytes *in vitro* in a consistently normal healthy condition for several days, Trowell had a reasonable background in which to look for cytotoxic effects. In a subsequent paper, Trowell (1953) showed that cortisone has a lethal action on rat lymphocytes in cultured lymph nodes. No specific cytological changes were found; the cells merely underwent a process of pycnotic degeneration, which the author regarded as the "non-specific autolysis of an already dead cell."

It would be of great interest to attempt organ cultures of the fish thymus, using the method of Trowell (1952). Perhaps in this manner a population of normal healthy thymocytes could be maintained *in vitro* long enough for the direct effects of the adrenal hormones to be determined. The many discrepancies in the literature may also be attributed to the fact that many different methods of approach have been used. According to Schrek (1949), the toxic action of adrenocortical steroids on lymphocytes is relatively mild, and, unless the method of testing is quite sensitive, the effect may be lost.

The various theories offered above, to explain the diverse views on the effects of adrenocortical hormones on lymphocytes *in vitro*, no doubt apply as well to our results. In addition, however, we must consider the possibility of hormone specificity. The teleost thymus under these circumstances may be refractory to mammalian hormones. Rasquin (1951) has shown that the thymus of *Astyanax* is not readily involuted by mammalian ACTH, although it is very responsive to implanted whole teleost pituitary. Injections of mammalian cortisone also failed to elicit any changes in the thymus of this teleost (Rasquin and Atz, 1952). A temperature factor may be one of the reasons for a lack of effect of mammalian adrenocortical hormones on teleost lymphoid tissue. Schrek (1949) reported that the adrenocortical hormones were effective only when the cells were incubated within a narrow temperature range. Since our cultures were of cold-blooded tissue, incubation was at room temperature, which may be below the effective range for these specific mammalian hormones.

It would certainly be of extreme interest to subject our thymus cultures to fish hormone preparations. Owing to the nature of the teleost anterior interrenal tissue, however, which is homologous with the mammalian adrenal cortex (Rasquin, 1951), extraction of the hormones is not feasible with existing methods.

There also appears to be little accord in the literature concerning the effects of the adrenal hormones on either epithelial or fibroblastic tissue *in vitro*. Our results on fibroblasts and

epithelial cells of the teleost thymus tissue are in agreement with those of Baldridge, Kligman, Lipnik, and Pillsbury (1951), who showed that proliferation of chick-embryo fibroblasts is not inhibited in tissue cultures containing cortisone. Steen (1951) also found that cortisone had no inhibitory effect on cultures of epithelial or fibroblastic tissue of chick embryos, in concentrations comparable to and even 25 times greater than usual therapeutic levels.

In reviewing the occurrence of spontaneous tumors in cold-blooded vertebrates, Schlumberger and Lucké (1948) and Lucké and Schlumberger (1949) showed that tumors of mesenchymal tissue are the most prevalent type in fishes. The lymphosarcoma has been reported in several species of fish. An isolated case of a spontaneous lymphosarcoma in *Astyanax mexicanus* was described by Nigrelli (1947) as probably having its origin in the thymus-like lymphoid gland. Four similar tumors have since appeared spontaneously in this same species in our laboratory. The description of the gross microscopic anatomy of the tumor (Rasquin and Hafter, 1951) corresponds to descriptions of lymphosarcoma in other fish, as well as for mammals as described by Boyd (1939) and Willis (1950; 1953). The tumor in *Astyanax* was not transplantable since subcutaneous, intramuscular, and intraperitoneal transplants (Rasquin and Rosenbloom, 1954) showed no signs of tumor development even after one year. Our material therefore was considerably limited.

Fixed and stained histological preparations of biopsy material from the two tumorous animals appeared similar, but in tissue culture the patterns of growth were quite different, with the exception of an initial migration of lymphocytes. In one, there was a proliferation predominantly of epithelial sheets, whereas in the other, fibroblasts predominated. It is conceivable that we are concerned with two different developmental stages of the same tumor, the latter showing a more advanced pathology. Grand (1949), in a study of lymph nodes with Hodgkin's disease in tissue culture, also observed a preponderance of different cell types, depending upon the degree of involvement of the nodes. Cultures of the more advanced tumors showed mostly fibroblasts.

The close functional relationship that exists between the pituitary, adrenal cortex, and normal lymphoid tissue in mammals and in *Astyanax* is known. Considerable attention has been given to the role of the adrenocortical hormones in either preventing or alleviating lymphoid tumors both experimentally and clinically (Heilman and Kendall, 1944; Soffer, Gabrilove, Laqueur, Volterra, Jacobs, and Sussman, 1948; Pearson, Eliel, Rawson, Dobriner, and Rhoads, 1949; Spies, Stone, Lopez, Milanes, Toca, and Roboredo, 1950; Wooley, 1950; Sugiura, Stock, and Rhoads, 1950; and Emerson, Wurtz, and Zanetti, 1950).

In teleosts, the same interrelationship exists between lymphoid disfunction and the pituitary and adrenal glands. A discussion of the significance of hormonal imbalance in relation to the development of hyperplasias as well as neoplasias (including lymphosarcoma) in teleosts is presented by Rasquin and Rosenbloom (1954). Rasquin and Hafter (1951) showed that the injections of ACTH into one of the lymphosarcomatous fish from which biopsy material was cultured for this report resulted in a marked dissolution of tumor elements. It is not surprising that the ACTH was without effect in tissue culture, since it is a trophic hormone. As concerns treatment with the various mammalian adrenal hormones, the reasoning applied to the lack of effect on the normal thymus tissue obtains as well. Possibly, if homologous hormones were used and a valid criterion for determining the results was established, more striking effects of adrenal hormones on malignant teleost lymphoid tissue *in vitro* could be observed.

SUMMARY

1. NORMAL AND MALIGNANT thymus tissues of the adult teleost *Astyanax mexicanus* were successfully grown in tissue culture for the first time, with the use of the double coverslip technique of Maximow.

2. Migration of thymocytes and thymoblasts was observed within the first few hours of *in-vitro* growth in the normal thymus. Other free cells that also made an early appearance included erythrocytes, small eosinophilic wandering cells, macrophages, and melanophores. Proliferation of epithelial reticular cells was usually observed between 36 and 48 hours after explantation, although occasionally some cells were seen as early as 24 hours. Multinucleation in the epithelial cells is described and discussed. Fibroblasts, present only in cultures of older fish, were the last cells to proliferate, usually after 48 hours. A description of these various cell types and their modes of migration or proliferation is presented. Comparisons are made between the normal thymus *in vivo* (through histological studies) and the thymus *in vitro*.

3. Histochemical determinations revealed that the epithelial cytoplasmic inclusions were predominantly lipid in nature (neutral fats and phospholipids). Some of the inclusions contained acid mucopolysaccharides. The possible significance of these results is discussed.

4. There were no clear-cut determinable effects of either mammalian cortisone or whole adrenal extract, in the dosages used, on the normal thymus *in vitro*. The lack of a valid criterion for such determinations in tissue cultures of thymocytes is discussed.

5. A description is given of the morphology in tissue culture of two spontaneous lymphosarcomas (reputedly of thymic origin), which occurred in our laboratory, in *Astyanax*. Comparison is made with normal thymus tissue. In general, the rate and extent of proliferation of cells in the tumor cultures far exceeded those in normal thymus cultures. With the limited data available, no valid conclusions can be drawn concerning the effects of various mammalian compounds (compound E, compound A, whole adrenal extract, and ACTH), on the lymphosarcoma *in vitro*.

BIBLIOGRAPHY

ACKERMAN, G. A., R. A. KNOUFF, AND H. A. HOSTER
1952. Cytochemistry and morphology of human lymph node cells grown in vitro. Jour. Natl. Cancer Inst., vol. 12, pp. 1267–1277.

ALLEN, C.
1954. The skin. A clinicopathologic treatise. St. Louis, C. V. Mosby Company.

ANDREASEN, E., AND J. OTTESEN
1945. Studies on lymphocyte production. Investigations on the nucleic acid turnover in the lymphoid organs. Acta Physiol. Scandinavica, vol. 10, pp. 258–270.

BAITSELL, G. A.
1924. Giant cells in cultures of amphibian tissues. Anat. Rec., vol. 29, pp. 105–106.

BAKER, J. R.
1946. The histochemical recognition of lipine. Quart. Jour. Micros. Sci., vol. 87, pp. 441–470.

BALDRIDGE, C. D., A. M. KLIGMAN, M. J. LIPNIK, AND D. M. PILLSBURY
1951. In-vitro effects of cortisone on mesodermal tissue. Arch. Pathol., vol. 51, pp. 593–596.

BARTA, E.
1926a. Deficient oxidation as a cause of giant cell formation in tissue cultures of lymph nodes. Arch. Exp. Zellforsch., vol. 2, pp. 6–30.
1926b. Les cellules géantes dans les cultures de tissus en rapport avec l'oxidation cellulaire et la formation de graisse intracellulaire. Compt. Rendus Soc. Biol. Paris, vol. 94, pp. 1182–1184.

BENSLEY, C. M.
1939. Comparison of methods for demonstrating glycogen microscopically. Stain Tech., vol. 14, pp. 47–52.

BLOOM, W.
1928. Mammalian lymph in tissue culture. From lymphocyte to fibroblast. Arch.

Exp. Zellforsch., vol. 5, pp. 269–307.
1937. Transformation of lymphocytes into granulocytes in vitro. Anat. Rec., vol. 69, pp. 99–122.

BOYD, W.
1939. A textbook of pathology. Third edition. Philadelphia, Lea and Febiger, pp. 833–838.

CAIN, A. J.
1947. An examination of Baker's Acid hematein test for phospholipids. Quart. Jour. Micros. Sci., vol. 88, pp. 467–478.

CATTON, W. T.
1951. Blood cell formations in certain teleost fishes. Blood, vol. 6, pp. 39–60.

COWDRY, E. V.
1952. Laboratory techniques in biology and medicine. Third edition. Baltimore, the Williams and Wilkins Co.

DEANE, H. W., F. B. NESBETT, AND A. B. HASTINGS
1946. Improved fixation for histological demonstration of glycogen and comparison with chemical determinations in liver. Proc. Soc. Exp. Biol., vol. 63, pp. 401–406.

DE BRUYN, P. P. H.
1944. Locomotion of blood cells in tissue culture. Anat. Rec., vol. 89, pp. 43–64.

DOMBROWSKI, H.
1953. Untersuchungen über das Blut des Karpfens (*Cyprinus carpio* L.) und einiger anderer Süsswasserfischarten. Biol. Zentralbl., vol. 72, nos. 3/4, pp. 182–195.

DOUGHERTY, T. F., AND A. WHITE
1945. Functional alterations in lymphoid tissue induced by adrenal cortical secretion. Amer. Jour. Anat., vol. 77, pp. 81–116.

EMERSON, G. A., E. WURTZ, AND M. E. ZANETTI
1950. Regression of lymphosarcoma transplants following the administration of cortisone to riboflavin-deficient mice. Fed. Proc., vol. 9, pp. 357–358.

EMMART, E. W.
1936. A study of the histogenesis of the thymus in vitro. Anat. Rec., vol. 66, pp. 59–73.

FISCHER, A.
1922. A three-months old strain of epithelium. Jour. Exp. Med., vol. 35, pp. 367–372.

FORKNER, C. E.
1930. The origin and fate of two types of multinucleated giant cells in the circulating blood. Jour. Exp. Med., vol. 52, pp. 279–298.

GOLDSTEIN, M. N.
1954a. Formation of giant cells from human monocytes cultivated on cellophane. Anat. Rec., vol. 118, pp. 577–592.
1954b. The desoxyribose nucleic acid (DNA) content of human monocytes and their derivatives during giant cell formation in vitro. Jour. Histochem. and Cytochem., vol. 2, pp. 274–281.

GRAND, C. G.
1949. Cytoplasmic inclusions and the characteristics of Hodgkin's diseased lymph nodes in tissue culture. Cancer Res., vol. 9, pp. 183–192.

GRAND, C. G., M. GORDON, AND G. CAMERON
1941. Neoplasm studies VIII. Cell types in tissue culture of fish melanotic tumors compared with mammalian melanomas. Cancer Res., vol. 1, pp. 660–666.

GREENBERG, S. S., AND M. J. KOPAC
1961. Melanogenic enzyme activity in the gene-induced tumors of xiphophorin fishes. Presented at Pigment Cell Conference. New York, 1961.

GREENBERG, S. S., M. J. KOPAC, AND M. GORDON
1956. Cytology and cytochemistry of melanoma cells. Ann. New York Acad. Sci., vol. 67, art. 4.

HAFTER, E.
1952. Histological age changes in the thymus of the teleost, *Astyanax*. Jour. Morph., vol. 90, pp. 555–582.

HEILMAN, D. H.
1945. The effect of 11-dehydro-17-hydroxycorticosterone and 11-dehydrocorticosterone on lymphocytes in tissue culture. Proc. Mayo Clin., vol. 20, pp. 310–312.

HEILMAN, F. R., AND E. C. KENDALL
1944. The influence of 11-dehydro-17-hydroxycorticosterone (compound E) on the growth of a malignant tumor in the mouse. Endocrinology, vol. 34, pp. 416–420.

HU, F., AND W. CHAVIN
1960. Hormonal stimulation of melanogenesis in tissue culture. Jour. Invest. Dermat., vol. 34, pp. 377–391.

HUEPER, W. C., AND M. A. RUSSELL
1932. "Capillary-like formations" in tissue cultures of leucocytes. Arch. Exp. Zellforsch., vol. 12, pp. 407–424.

JORDAN, H. E.
1925. Varieties and the significance of giant cells. Anat. Rec., vol. 31, pp. 51–64.

JORDAN, H. E., AND C. C. SPEIDEL
1924. Studies on lymphocytes. II. The origin, function and fate of the lymphocytes

in fishes. Jour. Morph., vol. 38, pp. 529–548.

KIM, K. H., T. T. TCHEN, AND F. HU
1961. Studies on the mechanism of ACTH-induced melanocyte formation in goldfish. Presented at Pigment Cell Conference. New York, 1961.

KRIPPAEHNE, M. L., AND R. R. OSGOOD
1955. Studies of the influence of cortisone and hydrocortisone on human leukocytes in culture and in eosinophilic leukemia. Acta Haemat., vol. 13, pp. 145–152.

LAMBERT, R. A.
1912. The production of foreign body giant cells in vitro. Jour. Exp. Med., vol. 15, pp. 510–515.

LANGHANS, T.
1868. Über Riesenzellen mit wandständigen Kernen in Tuberkeln und die fibrose Form des Tuberkels. Virchows Arch., vol. 42, pp. 382–404.

LEVI, G.
1934. Explantation, besonders die Struktur und die biologischen Eigenschaften der in vitro gezüchteten Zellen und Gewebe. Ergeb. Anat. u. Entwicklungsgesch., vol. 31, pp. 125–707.

LEWIS, M. R., AND W. H. LEWIS
1925. Monocytes, macrophages, epithelioid cells and giant cells. Anat. Rec., vol. 29, p. 391. (Abstract.)
1926. Transformation of mononuclear blood cells into macrophages, epithelioid cells and giant cells in hanging drop blood cultures from lower vertebrates. Contrib. Embryol. Carnegie Inst., vol. 96, no. 18, pp. 95–120.

LEWIS, W. H.
1925–1926. The transformation of mononuclear blood cells into macrophages, epithelioid cells and giant cells. Harvey Lectures. Baltimore, vol. 21, pp. 77–112.
1926. On the possibility of the transformation of polymorphonuclear leucocytes into mononuclear epithelioid cells and macrophages in cultures of buffy coat of the blood of the rat. Anat. Rec., vol. 32, p. 216. (Abstract.)

LEWIS, W. H., AND M. R. LEWIS
1925. The transformation of leucocytes into macrophages, epithelioid cells and giant cells in cultures of pure blood. Amer. Jour. Physiol., vol. 72, p. 196. (Abstract.)

LEWIS, W. H., AND L. T. WEBSTER
1921. Giant cells in cultures from human lymph nodes. Jour. Exp. Med., vol. 33, pp. 349–360.

LILLIE, R. D.
1944. Studies of certain oils, soluble dyes, for use as fat stains. Jour. Tech. Methods, vol. 24, pp. 37–42.
1948. Histopathologic technic. Philadelphia, the Blakiston Co.

LOEWENTHAL, L. A., AND C. SMITH
1952. Studies on the thymus of the mammal. IV. Lipid-laden foamy cells in the involuting thymus of the mouse. Anat. Rec., vol. 112, pp. 1–16.

LUCKÉ, B., AND H. G. SCHLUMBERGER
1949. Neoplasia in cold-blooded vertebrates. Physiol. Rev., vol. 29, pp. 91–126.

MACKLIN, C. C.
1916. Binucleate and multinucleate cells in tissue cultures. Anat. Rec., vol. 10, p. 225. (Abstract.)

MAXIMOW, A.
1924. Tuberculosis of mammalian tissue in vitro. Jour. Infect. Dis., vol. 34, pp. 549–584.

MENDELSOHN, W.
1954. The cultivation of rabbit thymus in roller tubes. Anat. Rec., vol. 120, pp. 73–84.

MUHLETHALER, J. P.
1952. Quelques observations sur des cultures in vitro en présence de bleu de trypan. Acta Anat., vol. 15, pp. 156–176.

MURRAY, M. R., AND G. KOPECH
1953. A bibliography of the research in tissue culture 1884–1950. An index to the literature of the living cell cultivated in vitro. New York, Academic Press, Inc.

MURRAY, R. G.
1947. Pure cultures of rabbit thymus epithelium. Amer. Jour. Anat., vol. 81, pp. 369–411.

NIGRELLI, R. F.
1947. Spontaneous neoplasms in fishes. III. Lymphosarcoma in *Astyanax* and *Esox*. Zoologica, New York, vol. 32, pp. 101–108.

PAPPENHEIMER, A.
1913. Studies on the histogenesis of the thymus. Amer. Jour. Anat., vol. 14, pp. 299–332.

PEARSE, A. G. E.
1960. Histochemistry. Theoretical and applied. Boston, Little, Brown and Co.

PEARSON, O. H., L. P. ELIEL, R. W. RAWSON, K. DOBRINER, AND C. P. RHOADS
1949. ACTH and cortisone-induced regression of lymphoid tumors in man. A preliminary report. Cancer, vol. 2, pp. 943–945.

PFEIFFER, H.
1935. Versuche über die Deformation kleiner Lymphozyten und Retikulumzellen aus der in vitro kultivierten Milz von *Petromyzon fluviatilis*. Arch. Exp. Zellforsch., vol. 17, pp. 456–462.

PIRES SOARES, J. M.
1937. Observations sur les cultures de leucocytes in vitro. Transformation des monocytes en macrophages, cellules géantes et cellule epithelioïdes. Compt. Rendus Soc. Biol., Paris, vol. 125, pp. 565–568.

POPOFF, N. W.
1927. The histogenesis of the thymus as shown by tissue cultures. Arch. Exp. Zellforsch., vol. 4, pp. 395–418.

PURVES, H. D., AND W. E. GRIESBACH
1951. The site of thyrotrophin and gonadotrophin production in the rat pituitary studied by McManus-Hotchkiss staining for glycoprotein. Endocrinology, vol. 49, pp. 244–264.

RASQUIN, P.
1951. Effects of carp pituitary and mammalian ACTH on the endocrine and lymphoid systems of the teleost, *Astyanax mexicanus*. Jour. Exp. Zool., vol. 117, pp. 317–358.

RASQUIN, P., AND E. H. ATZ
1952. Effects of ACTH and cortisone on the pituitary, thyroid and gonads of the teleost, *Astyanax mexicanus*. Zoologica, New York, vol. 37, pp. 77–87.

RASQUIN, P., AND E. HAFTER
1951. Response of a spontaneous fish lymphosarcoma to mammalian ACTH. Zoologica, New York, vol. 36, pp. 163–169.

RASQUIN, P., AND L. ROSENBLOOM
1954. Endocrine imbalance and tissue hyperplasia in teleosts maintained in darkness. Bull. Amer. Mus. Nat. Hist., vol. 104, pp. 359–426.

ROTTINO, A., AND A. HOLLENDER
1949a. In vitro studies of lymph nodes involved in Hodgkin's disease etc. I. Liquefaction of culture medium. Arch. Pathol., vol. 47, pp. 317–327.
1949b. In vitro studies of lymph nodes involved in Hodgkin's disease. II. Tissue culture studies; formation, behavior and significance of the multinucleated giant cells. *Ibid.*, vol. 47, pp. 328–334.

SALKIND, J.
1912. Sur l'organisation du thymus. Anat. Anz., vol. 41, pp. 145–155.

SCHLUMBERGER, H. G., AND B. LUCKÉ
1948. Tumors of fishes, amphibians and reptiles. Cancer Res., vol. 8, pp. 657–754.

SCHREK, R.
1949. Cytotoxic action of hormones of the adrenal cortex according to the method of unstained cell counts. Endocrinology, vol. 45, pp. 317–334.

SELYE, H.
1946. The general adaptation syndrome and the diseases of adaptation. Jour. Clin. Endocrinol., vol. 6, pp. 117–230.

SMITH, C., AND F. C. THOMAS
1950. Studies on the thymus of the mammal. III. Glycogen in the cortical cells of the thymus. Anat. Rec., vol. 106, pp. 17–29.

SOFFER, I. J., J. L. GABRILOVE, H. P. LAQUEUR, M. VOLTERRA, M. D. JACOBS, AND M. L. SUSSMAN
1948. The effects of anterior pituitary adrenocorticotropic hormone in myasthenia gravis with tumor of the thymus. Jour. Mt. Sinai Hosp., New York, vol. 15, pp. 73–82.

SPIES, T., R. E. STONE, G. G. LOPEZ, F. MILANES, R. L. TOCA, AND A. REBOREDO
1950. Response to adrenocorticotrophic hormone and cortisone in persons with carcinoma, leukemia and lymphosarcoma. Lancet, vol. 259, pp. 241–244.

STEEDMAN, H. F.
1950. Alcian blue 8GS: A new stain for mucin. Quart. Jour. Micros. Sci., ser. 3, vol. 91, pp. 477–479.

STEEN, A. S.
1951. Effect of cortisone on tissue cultures. Brit. Jour. Ophthal., vol. 35, pp. 741–749.

SUGIURA, K., C. C. STOCK, AND C. P. RHOADS
1950. The effect of cortisone and other steroids on experimental tumors. Cancer Res., vol. 10, pp. 244–245.

TAFT, E. B.
1951. The problem of a standardized technique for the MGP stain. Stain Tech., vol. 21, pp. 205–212.

TIMOFEJEWSKY, A., AND S. BENEWOLENSKAJA
1927. Tuberculous inoculation in cultures of leucocytes of the human blood. Arch. Exp. Zellforsch., vol. 4, pp. 64–78.

TROWELL, O. A.
1949a. Culture of lymphoid tissue in vitro. Jour. Physiol., vol. 110, nos. 1–2, p. 4P.
1949b. Intracellular lymphocytes in thymus reticular cells and in fibroblasts cultured in vitro. *Ibid.*, vol. 110, nos. 1–2, p. 5P.

1952. The culture of lymph nodes in vitro. Exp. Cell Res., vol. 3, pp. 79–107.

1953. The action of cortisone on lymphocytes in vitro. Jour. Physiol., vol. 119, pp. 274–285.

1958. Some properties of lymphocytes in-vivo and in-vitro. Ann. New York Acad. Sci., vol. 73, pp. 105–112.

TSCHASSOWNIKOW, N.

1927. Über die in vitro Kulturen des Thymus. Arch. Exp. Zellforsch., vol. 3, pp. 250–276.

VERNON, M. L.

1958. Experimental in-vitro studies of the mammalian thymus. Dissertation Abstr., Ann Arbor, vol. 19, p. 1427.

WASSEN, A. L.

1915. Beobachtungen an Thymuskulturen in vitro. Anat. Hefte, vol. 52, pp. 277–318.

WEIL, G. C.

1913–1914. Spontaneous and artificial development of giant cells in vitro. Jour. Pathol. Bact., vol. 18, pp. 1–7.

WEISS, L. P., AND D. W. FAWCETT

1953. Cytochemical observations on chicken monocytes, macrophages and giant cells in tissue culture. Jour. Histochem. and Cytochem., vol. 1, pp. 47–65.

WILLIS, R. A.

1950. The principles of pathology. London, Butterworth and Co., Ltd., p. 537.

1953. Pathology of tumors. Second edition. London, Butterworth and Co., Ltd., p. 771.

WOLF, KEN, AND C. E. DUNBAR

1957. Cultivation of adult teleost tissues in-vitro. Proc. Soc. Exp. Biol., vol. 95, pp. 455–458.

WOLF, KEN, M. C. QUIMBY, E. A. PYLE, AND R. P. DEXTER

1960. Preparation of monolayer cell cultures from tissues of some lower vertebrates. Science, vol. 132, pp. 1890–1891.

WOOLEY, G. W.

1950. 11-dehydrocorticosterone acetate (compound A) in normal and tumor bearing mice. Proc. Soc. Exp. Biol., New York, vol. 74, pp. 286–289.