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RADIOAUTOGRAPHIC AND HISTOLOGIC INVESTIGATION OF SKIN IN YOUNG AND OLD FROGS*

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ABSTRACT

Age differences in the skin structure have been studied in young (one year-old) and aged (five and a half year-old) frogs, *Xenopus laevis*. The epidermis in young frogs is made up of an average of 6.3 and 4.7 layers of epithelial cells at abdominal and dorsal surfaces, respectively. In aged frogs, the number of respective cell layers at abdominal and dorsal surfaces increases to 8.8 and 5.6. The thickness of the dermis (spongiosum) in aged frogs is decreased 25% on the abdominal side (from 267 μm to 207 μm) but is increased by 11% on the dorsal side (from 275 μm to 305 μm). The nucleolar index and ^{3}H-uridine incorporation, as judged by radioautography, by epithelial cells are drastically reduced in aged frogs.

Key Words:

Radioautography, ^{3}H-uridine, Frog, Epidermis, Skin

INTRODUCTION

Because of the physiologic characteristic of the frog skin as an effective diffusion barrier to ionic backflow from the surface, considerable efforts have been made in the exploration of their structure (Bourquet and Maetz, 1961[4], Engbaek and Hoshiko, 1957[8], Jorgensen, 1950[14], Koefoed-Johnson and Ussing, 1953[16]). Along with light microscopic studies of Engbaek and Hoshiko (1957)[8], and Scheer and Mumbach (1960)[24], electron microscopic work by Parakkal and Matoltsy (1964)[22], Voute (1963)[26], Budtz and Larsen (1973 [6] and 1975[7]), and Farquhar and Palade (1965)[9] have clarified the similarities and differences of the structure of amphibian skin when compared to those of mammals.

With the exception of certain features such as differences in stratum granulosum cells, the production of mucus and the persistence of the plasma membrane in outer cornified epithelial cells, the basic structural plan of amphibian skin is essentially the same as that observed in mammalian species. Farquhar and Palade (1965)[9], in their electron microscopic study of several different amphibian species (*Rana pipiens, Rana cateshiana, Bufo marinus, Xenopus laevis,* and *Amblystoma punctatum),* described that most frog skins consist of an epidermis composed of five to seven layers of epithelial cells and an underlying dermis, containing blood vessels, glands, and various fibers and cellular elements of the connective tissue. The epidermis has one or two layers of partially cornified, squamous cells, three to four intermediate layers of polyhedral cells (stratum granulosum and stratum spinosum), and a layer of cuboidal or columnar cells (stratum basale or stratum germinativum). Our survey of the literature failed to disclose any record of age-related differences in the structure of amphibian skin beyond the general description on metamorphosis and related changes in relatively young frogs.

In the past few years we have become interested in the amphibian skin as a potential new model for studies of cellular and molecular mechanisms of aging. As part of our preliminary efforts in this direction we have studied the comparative structural features of skin in young and old frogs of a common aquatic species, *Xenopus laevis.* Because of the significant differences in appearance and thickness of epidermis between young and old frogs, our preliminary experiments were extended further to include a radioautographic assessment of general metabolic activities of epidermal cells in young and old *Xenopus laevis.* For this purpose, the incorporation of ^{3}H-uridine into epidermal cell nuclei was studied as this technique permits histologic evaluation of the site of RNA synthesis in geographically heterogeneous structures, and has been used in a large number of previous publications including our own (Han, Cho, Cohen and Keyes, 1977)[11]. This article communicates results of these experiments.

MATERIALS AND METHODS

Histological Preparation. *Xenopus laevis,* reared at the Amphibian Facilities of the University of Michigan, were used throughout this study. Male animals of one and five and a half years

of age were studied. They were placed under observation and were sacrificed during a 24 hour period following molting. Pieces of the skin were taken from the central area of the back and abdomen, fixed in ½ strength Karnovsky's (diluted by 0.2 M Millonig's PO^4 buffer, pH 7.3) solution, and embedded in a mixture of Araldite resin in the routine manner (Hayat, 1970)[13]. Following polymerization, the sample tissues were so oriented that histological sections prepared would represent a perfect lateral profile of the different layers of skin. The sections 1 μm in thickness were made on a LKB type 4801 A ultramicrotome, and were stained with toluidine blue. The mean thickness of epidermis and that of dermis was determined by multiple measurements of the sections using an ocular micrometer on a Zeiss photomicroscope. Because of the clear demarcation between the spongiosum and compacta layers of frog dermis and because of the lack of age-dependent difference in thickness of the stratum compacta, the thickness of the dermis described herein represents that of the stratum spongiosum only. The arithmetic means of these measurements from different individual animals were statistically analyzed using student t test. Therefore, the range of standard error of means and p values described in this report reflect individual variations within the age group.

Radioautographic Methods. In order to assess the general level of metabolic activities in cutaneous cells, pieces of the skin from frogs of the two age groups were incubated in amphibian physiologic saline containing 50 uCi/ml of 5-^{3}H-uridine with a specific activity of 25.9 Ci/mmole (New England Nuclear). The labeling was carried out for 30 minutes at 20 ± 1°C. At the conclusion of labeling the tissues were fixed and embedded in Araldite as described previously. Sections 1 μm in thickness were cut on a LKB type 4801 A ultramicrotome and mounted on 1 by 3 inch glass slides in such a manner so that sections from 18 blocks representing 3 animals in each of the age groups were mounted and could be coated for radioautography simultaneously (Han and Kim, 1972)[12]. This allows a uniform coating and identical processing of tissues during radioautographic exposure and development so that later efforts for grain counting would have a minimum variation resulting from the radioautographic procedure. The mounted slides were coated with Kodak NTB-3 nuclear track emulsion in a routine manner (Messier and Leblond, 1957)[18] exposed for a period of 1 or 2 weeks while kept in a refrigerator at 4°C, and were developed with Kodak D-19. Following development, the tissues were minimally stained with toluidine blue at room temperature and studied under an oil immersion objective with a Zeiss photomicroscope.

RESULTS AND OBSERVATIONS

Histology of young frog skin from the abdominal surface showed that the epidermis was made up of an average of 6.3 cell layers (Table 1 and Fig. 1); The epidermis frequently was interrupted by the straight ducts of underlying mucous and caerulein-producing glands (Fig. 1). Most of these glands were located in the stratum spongiosum of the dermis which was supported

Table 1. Epidermis of Skin in Young and Old Frogs[a]

– Xenopus laevis –

Age	Number of Cell Layers			Thickness (μm)		
	Age in Years		Ratio	Age in Years		Ratio
Region	1	5½	5½/1	1	5½	5½/1
Abdomen	6.3 (±0.2)	8.8 (±0.2)	1.40	75 (±3)	108 (±4)	1.44
Back	4.7 (±0.1)	5.6 (±0.2)	1.19	50 (±2)	54 (±3)	1.08

Note: [a]Numbers in parentheses denote standard error of means.
Numbers between young and old frogs are significant at P 0.05 or better.

Table 2. Dermis (Spongiosum) Thickness in Young and Old Frogs[a]

– Xenopus Laevis –

	Age in Years		Ratio
Region	1	5½	5½/1
Abdomen	267 (±9)	207 (±8)	0.78
Back	275 (±8)	305 (±8)	1.11

Note: [a] Thickness expressed in μm with standard error of means in parenthesis.
Numbers between young and old frogs are significant at P 0.05 or better.

by a relatively uniform layer of regularly arranged collagenous fiber mass of the stratum compacta which was unaffected by the age-difference.

The abdominal skin of old frogs showed an epidermis that was considerably thickened as indicated in Figure 2, which was magnified at the same magnification as Figure 1. The number of cell layers in the epidermis of five and a half year-old frogs was 8.8 which represented an increase of 40% over the thickness of the same region in one year-old frogs.

When the thickness of the epidermis was measured, the mean thickness in the young frogs measured to be 75 μm, whereas the same measurement had a mean value of 108 μm among the old frogs (Table 1 and 2). This represents 44% increase in the thickness of ventral epidermis among the old frogs.

The epidermis of skin on the dorsal side was generally thinner than that of the ventral side in both age groups. The average number of cell layers in the young frogs was 4.7, whereas in the old frogs the number was increased to 5.4 layers. The average thickness of the epidermis on the back increased from 50 μm to 54 μm between young and old age groups. This represents a 19% increase in the number of cell layers and an 8% increase in epidermal thickness. These increases are in contrast to the 40% and 44% differences in respective number seen on the abdominal side.

On the other hand, the thickness of the dermis (spongy layer) was reduced from an average of 267 μm in the young to an average of 207 μm to older animals which amounted to only 78% of the thickness of the young frogs (Table II). Histological observation indicated that the caerulein-producing glands of the dermis in the old frogs was decreased in size (cf. Figs. 1, 2 and Figs. 3, 4), whereas the mucus-secreting glands in the old animals were as numerous in number but had a more widely varied appearance than in the young frogs (cf. M in Fig. 1 with M_1, M_2 and M_3 in Fig. 2).

The spongy layer of dermis on the dorsal side was measured to be an average of 275 μm in the young which was increased to 305 μm in the old frogs. Thus, the thickness of the spongy layer of dermis shows an 11% increase on the dorsal side as compared to a 25% decrease on the ventral side.

When the relative epidermal thickness of young and old frogs were compared in these two regions, the abdominal side showed that the epidermis occupied 21.9% of the total thickness of the skin in the young. This was increased to 34.3% of the total thickness in the old representing 57% increase (Table III). In contrast, the relative epidermal thickness on the back remained more less at a constant level. The epidermis amounted to 15.4% and 15.0% of the dorsal skin in the young and old, respectively (Table III).

Despite of the fact that the frogs were killed during a comparable post molting phase, the cytology of epidermal cells between the young and old animals showed marked differences in both of the regions studied. Compared to the young, the cornifying cells of the abdominal surface in the old had a somewhat more irregular surface and were stained darker with toluidine blue in old frogs (cf. Figs. 1, 2 and Figs. 5, 7). Cells of the basal layer in the old also appeared to stain darker, in this case both in abdominal and dorsal surfaces (cf. Figs. 5, 7 and Figs. 9, 11). They also showed a more pronounced intercellular space in the old when compared to young frogs.

The number of nucleoli was reduced in old epidermal cells (Figs. 5, 7, 9 and 11). This was particularly so among the basal cells (Table IV). In the young, an average of 53% of the basal cells and 20% of the intermediate cells contained prominent nucleoli. These figures were reduced to 13% and 10%, respectively, in old frogs. The epidermal cells of the dorsal skin revealed that 30% of the basal cells and 21% of spinosum cells contained nucleoli. The percentage of cells with nucleoli was decreased by 17% among the basal cells in older animals, whereas those in the spinosum layer did not show any significant change.

Table 3. Relative Epidermal Thickness in Young and Old Frogs
— Ratio of Epidermis to Skin x100 (Xenopus laevis) —

	Age in Years		Ratio
Region	1	5¼	5¼/1
Abdomen	21.9	34.3	1.57
Back	15.4	15.0	0.97

Table 4. Nucleolar Index in Epidermal Cells in Young and Old Frogs[a]

— Xenopus laevis —

		Age in Years		Ratio
Region	Layer	1	5½	5½/1
Abdomen	Basal	53.1 (±4.8)	13.0 (±4.1)	0.24
	Spinous	19.8 (±1.7)	10.0 (±0.4)	0.50
Back	Basal	30.3 (±5.6)	16.6 (±2.9)	0.83
	Spinous	20.6 (±2.6)	19.6 (±1.4)	0.95

Note: [a] Numbers denote percentage of cells with nucleoli and standard error of means in parenthesis. Numbers between young and old frogs are significantly different (P 0.01) except for the nucleolar indices in the spinous layer of the back.

Table 5. ^{3}H-Uridine Incorporation by Epidermal Cells in Young and Old Frogs[a]

— Xenopus Laevis —

		Age in Years	
Region	Layer	1	5½
Abdomen	Basal	40.4 (±23.3)	0.0 (±0.0)
	Spinous	24.6 (± 4.0)	4.0 (±0.3)
Back	Basal	50.8 (±14.9)	0.0 (±0.0)
	Spinous	27.5 (± 6.3)	2.2 (±2.3)

Note: [a] Number denote percentage of labeled cells with standard error of means in parenthesis.

The grain localization in radioautographs revealed enough numbers of grains in young skin after an exposure period of one week. The radioautographs appearing Figures 6 and 10 represent results obtained from sections that were adjacent to Figures 5 and 9 which depict, respectively, ventral and dorsal epidermis of young frogs. However, the grain numbers over the epidermal cells of old animals were almost negligent after one week of exposure, despite of the fact that the tissues from old frogs were treated identically with those from the young ones in that all tissues were mounted on the same slide in which the emulsion layer was determined to be of uniform thickness. The results of grain counts for the old frogs, therefore, represent the ones that were taken from radioautographs exposed for two weeks. For this reason, a direct comparison of grain numbers or the percentage of labeled cells between young and old animals is not meaningful. The basal cells were unlabeled, while only 4% and 2.2% of spinosum layer cells were labeled even with a two-week exposure period (Table V). Additional experiments using longer exposure periods and higher specific activity precursors are in progress. The present results,

however, indicate strongly that basic biochemistry among epithelial cells between young and old frogs might be greatly different.

DISCUSSION

Salient and new aspects of the results obtained from the present study are that there are significant differences in the structure of epidermis between young and old frogs, and that such age-dependent differences are not the same in all areas of the frog skin. As pointed out elsewhere, the numerous publications on the histology and ultrastructure of amphibian skins in the past have failed to note regional differences in the appearance of the skin in any age groups. Nor has anyone described differential changes that occur in different regions of the amphibian skin as function of aging. The observed changes in the number of cell layers and the thickness of epidermis between young and old frogs, particularly those changes that occur at the ventral surface, presents an unique opportunity for exploring the reasons why such regional variations could take place with age and, therefore, add another dimension to the use of frog skin as model in the studies of cellular and molecular mechanism(s) of aging.

It was after a long and hard thinking that we adopted the cutaneous tissue as our primary model for inquiries into the basic biologic mechanisms of aging (Han, 1976)[10]. This was based on our belief that the best way of testing Hayflickian observations *in vivo* would be to study those renewing cells in which the dynamics of replicating cells could be established with certainty. It is recognized that research involving epidermal cell cycle determinations is expensive in terms of time as well as the amount of effort needed to generate data (Leblond, Grenlich and Pereira, 1964[17], Pirbazari, 1980[23], Thrasher, 1966)[25]. Along with lymphoid and hemopoietic cells, however, epithelial cell populations constitute one of the best systems in which possible changes in the expression of genomes could be studied, be they caused by aging or any other functional modifications. Epidermal cells would allow us to study modifications of both replicative and differentiative potentials, as the rate of marker molecule synthesis which appear during differentiation could be studied with a high degree of certainty (Ball, Walker and Bernstein, 1978)[1]. The synthesis of histidine-rich and sulfur-rich proteins has served as satisfactory molecular yardsticks in recent years (Bernstein, 1982)[2].

It might also be pointed out that frog skins could be used in experiments aimed at exploring the effect of accelerating chronological events by blocking hibernation. For example, frogs have been shown to go through upto three reproductive cycles per year, when they were kept from hibernation (Nace, 1980)[21]. It is our hope that by developing a frog model for cutaneous biology of aging we might eventually be able to compare the results from continuous active vs dormant epidermal cells in terms of their ability to continue replication and differentiation.

Results of this study indicate that there are significant differences in the structure of the skin between the ventral and dorsal sides of *Xenopus laevis* in both ages that were studied. The age difference in thickness of the epidermis is greater on the abdominal side, showing nearly 50% increase in old frogs from that of the young. This increase in epidermal thickness is achieved

by a corresponding increase in the number of cell layers, which might reflect the continuing adaptation of the abdominal skin throughout life to greater abrasive forces acting upon it than on the dorsal side. Although the number of cell layers in the dorsal side was increased 15% in the old animals, the increase in the thickness of epidermis amounted to only 8%. The 22% decrease in the thickness of the spongy layer of the dermis on the abdominal side might also reflect the effect of continuous physical pressure on the dermal glands of the skin. The dermis on the dorsal surface of the old frogs actually showed an 11% increase over that of the young ones.

The differences between young and old frogs in the radioautographic grains resulting from ^{3}H-uridine incorporation into the nuclei of epidermal cells are so great that twice as long an exposure time of radioautographs has failed to provide us with satisfactory grain numbers in the old animals. We could only tentatively conclude that in old frogs the epidermal cells synthesize RNA at a rate that may be a fraction of the synthetic rate in the young. Our labeling procedures and radioautographic methods have repeatedly been proven to be adequate in determining geographic and intracellular differences of cellular RNA synthesis (Han, Cho, Cohen and Keyes 1977[11], Kim, Corpron, Morawa and Han, 1982[15]).

In this connection there are a number of unresolved questions. For example, the significance of the relatively lower grain numbers over basal cells those over the spinosum layer is unknown. Nor do we understand the reason(s) why there is discrepancy between the drastic age-related difference in grain numbers and a moderate change in nucleolar indices, particularly in the dorsal skin.

Our results are not completely free from the interference that might result from possible age-related differences in molting cycle. Although we have taken samples at comparable post-molting periods, information regarding the age-specific differential effects of molting on epithelial cell renewal and/or their RNA synthesis is not available. In addition, the preliminary study, while noting a more active incorporation of ^{3}H-uridine into flask cells of older epidermis than into those of young frogs, could not offer any reason(s) for the difference. Along with other features such as the biology of dermal glands, the points mentioned above await for further, in-depth experimental inquiries of the future. The current literature recognizes two types of glands in the spongiosum layer of the dermis, i.e., caerulein-secreting and mucous glands. However, the heterogeneity in the architecture and staining quality of the mucous glands suggests that further characterization of these glands would be necessary before any descriptions of their age-dependent changes could be made.

In contrast to the popular concern over the age-changes of skin and dermatologic problems encountered among the elderly (Brucklehurst, 1973)[5] studies of the age-associated changes of skin has been somewhat limited (Montagna, 1965[19], Montagna and Carlisle, 1979[20]). Studies of amphibian skin with respect to aging have also been sparse. In light of the above, it is our belief that the results from our preliminary efforts provide us with a new opportunity of establishing an *in vivo* experimental model for basic research in cellular mechanism(s) of aging, and at the same time would allow us to explore the nature of age-related changes in cutaneous structures which may be relevant to our understanding of the skin changes during human aging.

SUMMARY

In this investigation the following observations are made:

1. The epidermis in young frogs has 6.3 cell layers on abdominal and 4.7 cell layers on dorsal sides.
2. In aged frogs the number of epidermal cell layers is increased to 8.8 and 5.6, respectively.
3. The dermal thickness in old frogs decreased on the abdominal side and increased on the dorsal side.
4. The nucleolar index and ^{3}H-uridine incorporation are drastically reduced in old frogs.

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Fig. 1. An Araldite-embedded, 1 μm-thick section of the abdominal skin of a young (one 1 year old) frog stained with toluidine blue. The epidermis is made up an average of 6.3 layers of epithelial cells (see text). The spongiosum layer of the dermis shows portions of two large caerulein glands and a mucous galnd (M) which has a straight duct opening through the epidermis. Magnification 225 X.

Fig. 2. An Araldite-embedded, 1 μm-thick section of the abdominal skin of an aged (five and a half year old) frog stained with toluidine blue. The epidermis is considerably thicker than in the young animal (8.8 cell layers). In the spongiosum layer of dermis mucous glands show varied appearances (M_1, M_2 and M_3). Magnification 225 X.

Fig. 3. An Araldite-embedded, 1 μm-thick section of the dorsal skin of a young frog stained with toluidine blue. The epidermis is thinner than the abdominal skin of frogs of the same age group and is made up of an average of 4.7 layers of epithelial cells. Mucous glands of the dermis are more numerous and are made up of two different varieties (M_1 and M_2). Pigments (arrows) present within the cytoplasm of dermal chromatophores appear dark. Magnification 225 X.

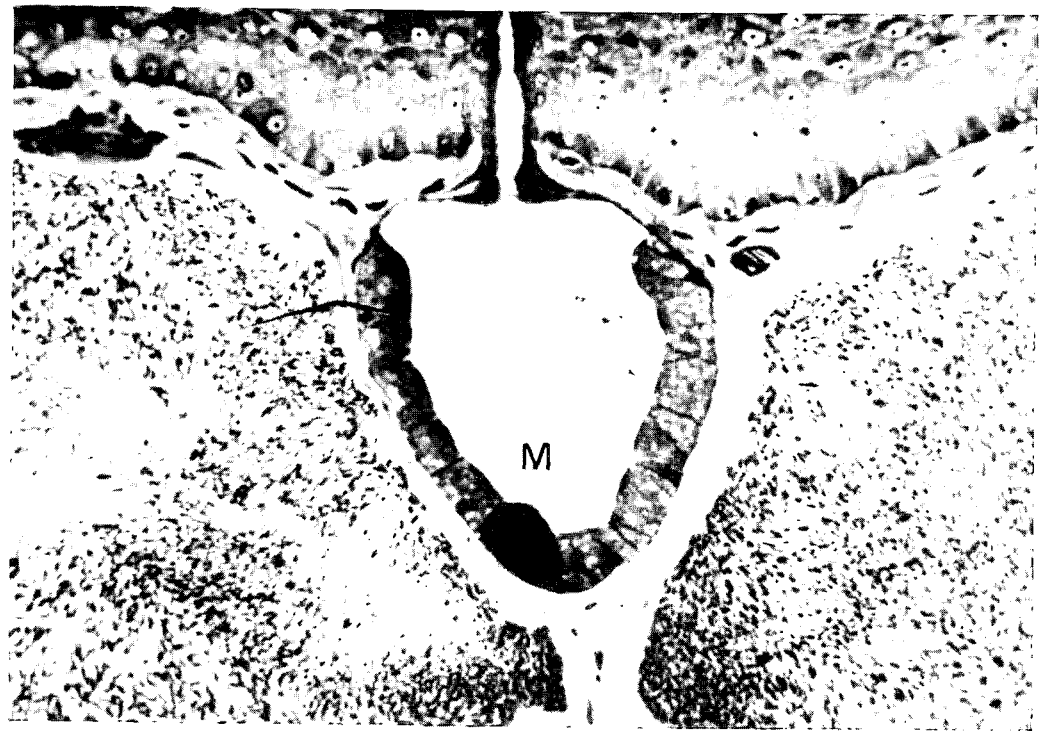
Fig. 4. An Araldite-embedded, 1 μm-thick section of the dorsal skin of an aged frog stained with toluidine blue. The marked increase of the epidermal thickness observed in the abdominal skin of aged frogs is not observed on the dorsal skin. The epidermis is only slightly thicker than the young frog and is made up of an average of 5.6 layers of epithelial cells. Magnification 179 X.

Fig. 5. A high magnification picture of the abdominal skin of a young frog embedded in Araldite, sectioned at 1 μm in thickness and stained with toluidine blue. Many epithelial cells have one or more nucleoli. Magnification 448 X.

Fig. 6. A radioautograph of a section adjacent to the one appearing in Figure 5. The tissue was incubated *in vitro* with ^{3}H-uridine for 30 minutes prior to fixation (see text). A large number of epidermal nuclei (25-40%) are labeled with more than 5 grains per nucleus (circled). Magnification 448 X.

Fig. 7. A high magnification picture of the abdominal skin of an aged frog embedded in Araldite, sectioned at 1 μm in thickness and stained with toluidine blue. The number of cells with nucleoli is less than in young frogs. Magnification 448 X.

Fig. 8. A radioautograph of a section adjacent to the one appearing in Figure 7. The tissue was incubated *in vitro* with ^{3}H-uridine for 30 minutes prior to fixation. Only a small number of epidermal cells (4%) is overlain by more than five grains per nucleus (circled). Magnification 448 X.



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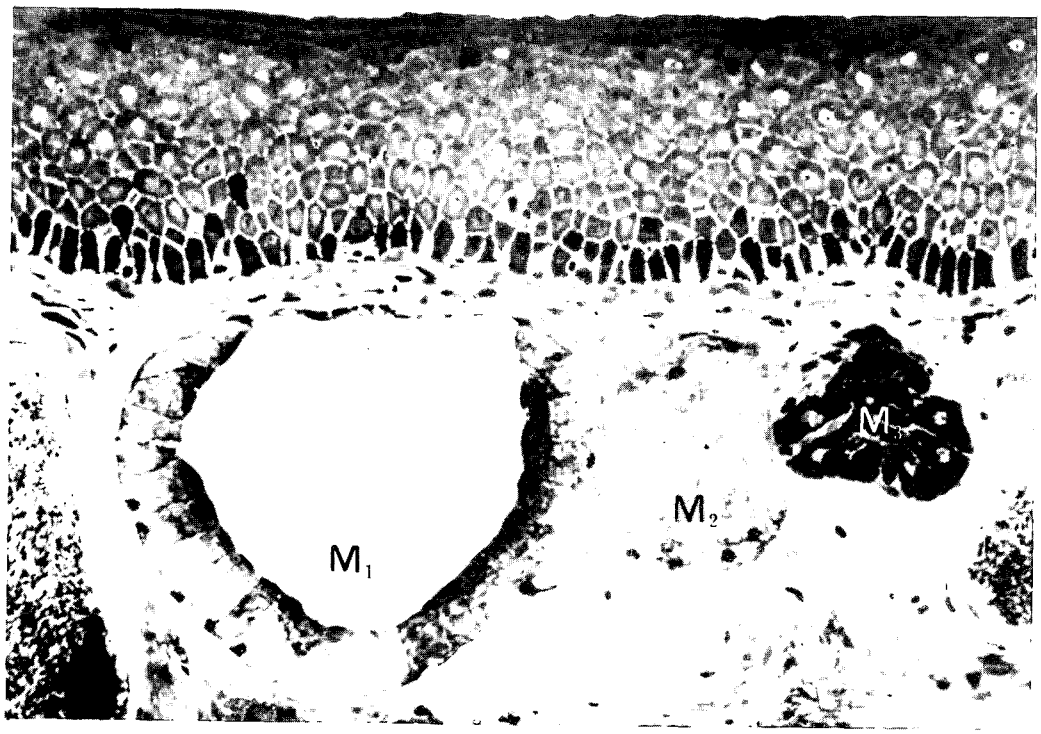

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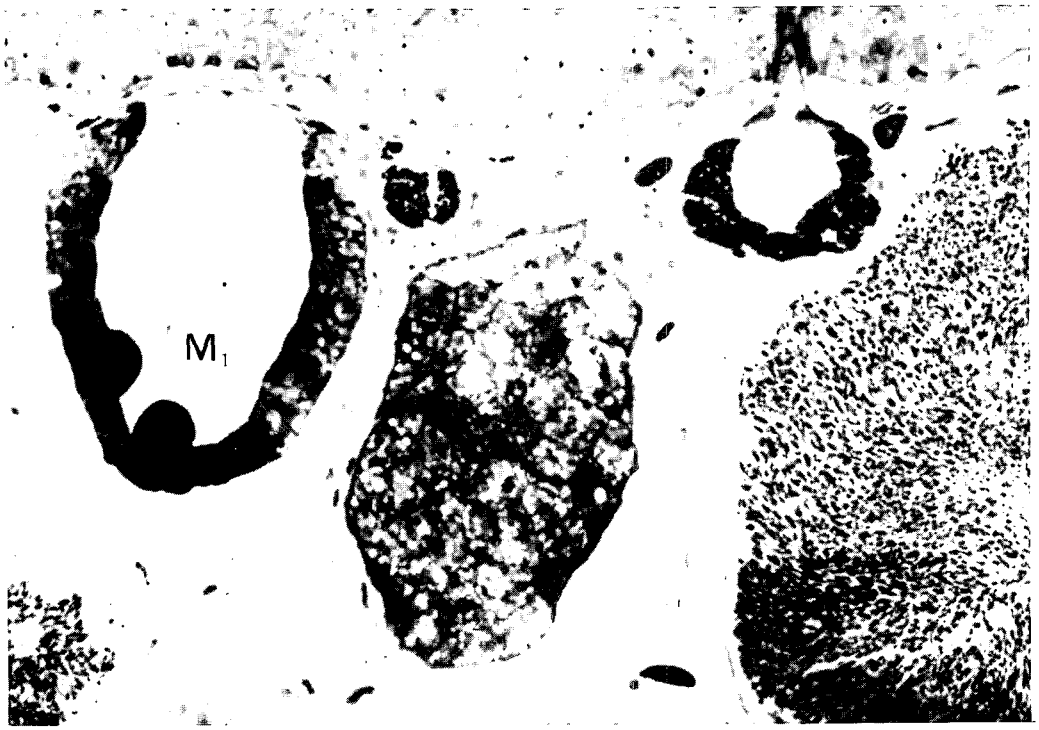

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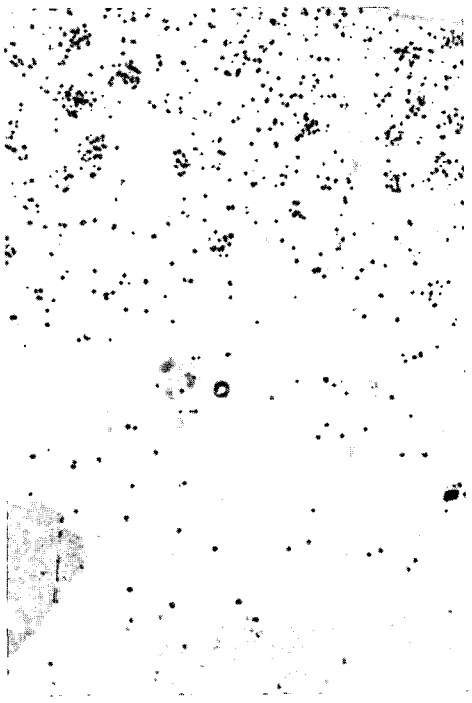

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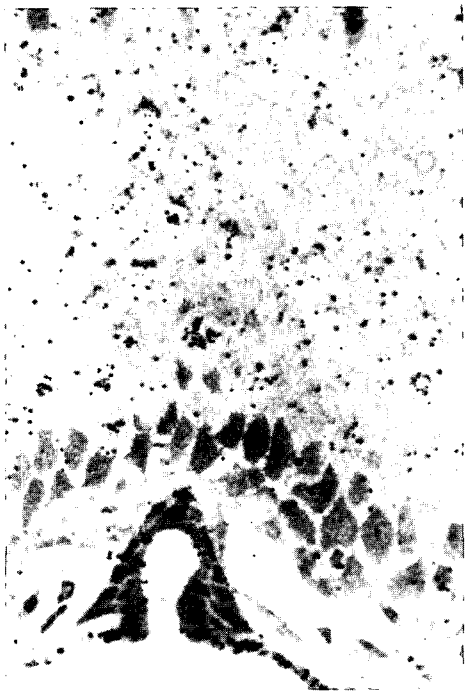

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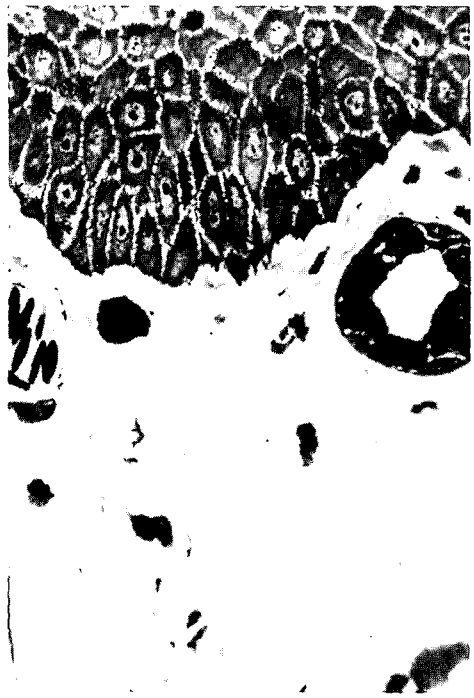

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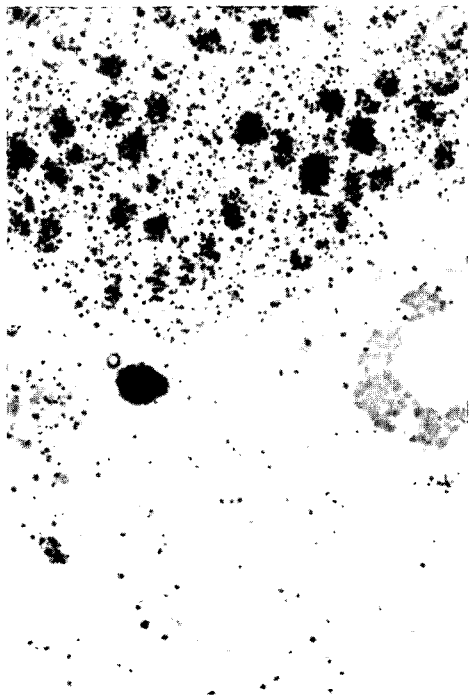

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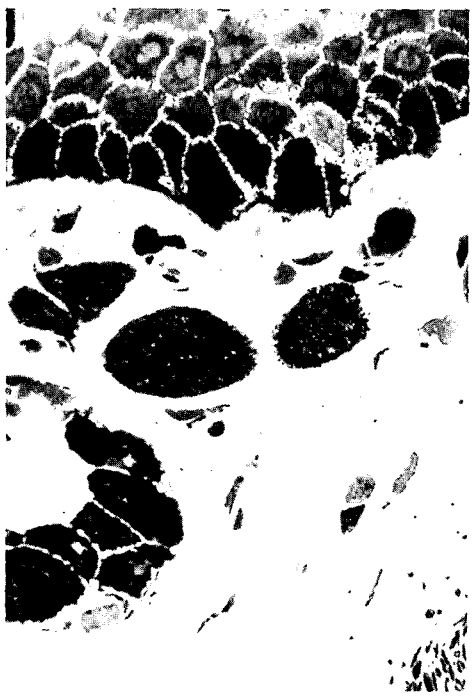

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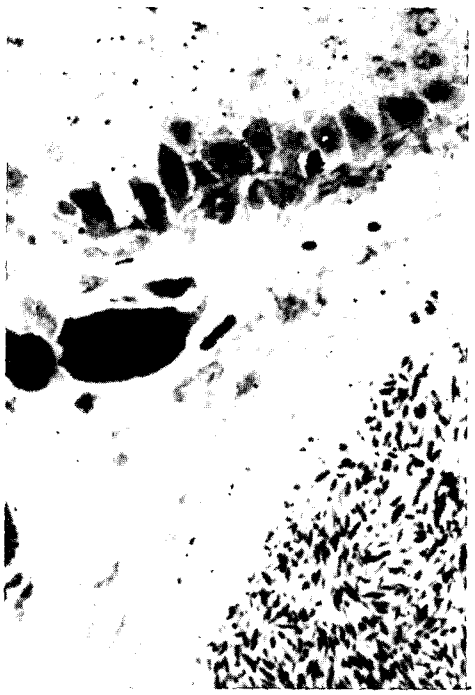

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