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Original Article

Electron microscopic radioautographic study on mitochondrial RNA synthesis in adrenal cells of aging mice

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ABSTRACT

In order to study the aging changes of intra mitochondrial RNA synthesis of mouse adreno-cortical and adreno-medullary cells, 10 groups of developing and aging mice, each consisting of 3 individuals, total 30, from fetal day 19 to postnatal newborn at day 1, 3, 9, 14, adult at month 1, 2, 6 and senescent animals at month 12 (year 1) and 24 (year 2) were injected with ^3H -uridine, an RNA precursor; sacrificed 1 hr later and the adrenal tissues were fixed and processed for electron microscopic radioautography. On electron microscopic radioautograms obtained from each animal, the number of mitochondria per cell, the number of labeled mitochondria with ^3H -uridine showing RNA synthesis per cell and the mitochondrial labeling index in each adreno-cortical cell, in 3 zones, as well as in each adreno-medullary cell, adrenalin cells and noradrenalin cells, were counted and the results in respective aging groups were compared with each others. From the results, it was demonstrated that the number of mitochondria per cell in 3 zones, the zona glomerulosa, fasciculata and reticularis of respective mice at various ages increased from fetal day 19 to postnatal month 1 reaching the plateau from month 1 to 24 due to development and aging of animals, respectively, while the number of labeled mitochondria per cell and the labeling index of intramitochondrial RNA synthesis incorporating ^3H -uridine increased from fetal day 19 to postnatal month 2, reaching the maxima and decreased slightly from month 6 to month 24. The mitochondrial labeling index calculated from the numbers of mitochondria per cell and the numbers of labeled mitochondria increased from fetal day 19 to postnatal day 14, reaching the maxima and decreased gradually from month 1 to month 24. On the other hand, the number of mitochondria in respective 2 types of cells, adrenalin and noradrenalin cells, increased from fetal day 19 to postnatal month 1, reaching the plateau from month 1 to 24 due to development and aging of animals, respectively, while the number of labeled mitochondria per cell and the labeling index of intramitochondrial RNA synthesis incorporating ^3H -uridine increased from fetal day 19 to postnatal month 2, reaching the maxima and decreased slightly from month 6 to month 24. Thus, the mitochondrial labeling index calculated from the numbers of mitochondria per cell and the numbers of labeled mitochondria increased from fetal day 19 to postnatal day 14, reaching the maxima and decreased gradually from month 1 to month 24. In conclusion, it was shown that the activity of intramitochondrial RNA synthesis in the adreno-cortical cells and adreno-medullary cells in developing and aging mice changed due to aging of individual animals.

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1. Introduction

Intramitochondrial nucleic acid syntheses, both DNA and RNA, in mammalian and avian cells were first demonstrated morphologically by the present author by means of cytochemistry

using electron microscopic radioautography in primary cultured cells of the livers and kidneys of mice and chickens in vitro [1, 2] and then in some other established cell lines such as HeLa cells [3-6] or mitochondrial fractions prepared from in vivo cells [7-9]. It was later commonly found in various cells and tissues not only in vitro obtained from various organs in vivo [10-14], but also in vivo cells of various organs such as the salivary glands [15], the liver [16-29], the pancreas [30, 31], the trachea [32], the lung [33], the kidneys [34], the testis [35, 36], the uterus [37, 38], the adrenals [39, 40, 41], the brains [42], and the retina [43-47] of mice, rats and chickens. The relationship between the intramitochondrial

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DNA synthesis and cell cycle was formerly studied and it was clarified that the intramitochondrial DNA synthesis was performed without nuclear involvement [3]. However, the relationship between the aging of individual animals and the DNA synthesis in respective cell types in these organs has not yet been clear. Recently, the relationship of both the DNA synthesis and RNA synthesis to the aging of animals was first clarified in the hepatocytes of mice [1, 2]. Later, the relationship of the DNA synthesis to the aging of animals in the adreno-cortical cells was also clarified [41]. However, the relationship of the RNA synthesis to the aging of animals in the adreno-cortical and adreno-medullary cells has not yet been clarified. This paper deals with the relationship between the RNA synthesis and the aging in the adrenal cortical and medullary cells of mice *in vivo* at various developmental stages from fetal day 19 to postnatal month 2 and further to adult and senescent stages up to month 24 (year 2) during aging by means of electron microscopic radioautography as a part of serial studies on special cytochemistry [48] and radioautographology [49].

2. Materials and Methods

2.1. Animals

The adrenal tissues were obtained from 10 groups of developing and aging normal ddY strain mice, from fetal day 19 to postnatal newborn at day 1, 3, 9, 14, adult at month 1, 2, 6, 12 and 24, each consisting of 3 litter mates of both sexes, total 30. The embryonic age was based on observation of the vaginal plug of the female mice (vaginal plug=day 0). All the animals were housed under conventional conditions and bred with normal diet (mouse chow Clea EC2, Clea Co., Tokyo, Japan) with access to water *ad libitum* in our laboratory. They were administered with ³H-uridine, RNA precursor, and the adrenal tissues were fixed and processed for electron microscopic radioautography. All the procedures used in this study concerning the animal experiments were in accordance with the guidelines of the animal research committee of Shinshu University School of Medicine, Matsumoto, Japan, where this experiment was carried out, as well as the principles of laboratory animal care in NIH publication No. 86-23 (revised 1985).

2.2. Electron Microscopic Radioautography

All the animals were injected intraperitoneally with ³H-uridine (Amersham, England, specific activity 877 GBq/mM) in saline, at 9 a.m., one hour before sacrifices. The dosage of injections was 370 KBq/gm body weight. The animals were perfused at 10 a.m., one hour after the injection, via the left ventricles of the hearts with 0.1 M cacodylate-buffered 2.5% glutaraldehyde under Nembutal (Abbott Laboratories, Chicago, ILL, USA) anesthesia. The right adrenal glands were taken out, excised and 3 small pieces of the adrenal tissues (1mm x 1mm x 1mm) were immersed in the same fixative at 4°C for 1 hr., followed by postfixation in 1% osmium tetroxide in the same buffer at 4°C for 1 hr., dehydrated in graded series of ethanol and acetone, and embedded in epoxy resin Epok 812 (Oken, Tokyo, Japan).

For electron microscopic radioautography, semithin sections at 0.2µm thickness, thicker than conventional ultrathin sections containing more radiolabeled compound than ultrathin sections in order to shorten the exposure time, were cut in sequence on a Porter-Blum MT-2B ultramicrotome (Dupont-Sorvall, Newtown, MA, USA) using glass knives. The sections were collected on collodion coated copper grid meshes (VECO, Eerbeek, Netherlands), coated with Konica NR-H2 radioautographic emulsion (Konica, Tokyo, Japan) by a wire-loop method [30, 48,

49, 50, 51]. They were stored in dark boxes containing silica gel (desiccant) at 4°C for exposure. After the exposure for 10 months, the specimens were processed for development in freshly prepared gold latensification solution for 30 sec at 16°C and then in fresh phenidon developer for 1 min at 16°C in a water bath, rinsed in distilled water and dried in an oven at 37°C overnight, stained with lead citrate solution for 3 min, coated with carbon for electron microscopy. The electron microscopic (EM) radioautograms were examined in a JEOL JEM-4000EX high voltage electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 400kV for observing thick specimens [49].

2.3. Quantitative Analysis of Electron Micrographs

For quantitative analysis of electron micrographs, twenty EM radioautograms showing cross sections of adreno-cortical cells sectioned through the centers of their nuclei and cell bodies selected at random from each group, based on the electron microscopic photographs taken after observation on at least 100 adreno-cortical cells from respective animals, and at least 10 cells from respective zones, i. e. zona glomerulosa, zona fasciculata and zona reticularis, were analyzed to calculate the total number of mitochondria in each adreno-cortical cell in respective zones, and the number of labeled mitochondria covered with silver grains by visual grain counting.

On the other hand, the number of silver grains in the same area size as a mitochondrion outside cells was also calculated in respective specimens as background fog, which resulted in less than 1 silver grain (0.03/mitochondrial area) almost zero. Therefore, the grain count in each specimen was not corrected with the background fog. From all the data thus obtained the averages and standard deviations in respective aging groups were computed with a personal computer (Macintosh type 8100/100, Apple Computer, Tokyo, Japan). The data were stochastically analyzed using variance and Student's t-test. The differences were considered to be significant at P value <0.01.

Likewise, twenty EM radioautograms showing cross sections of adreno-medullary cells sectioned through the centers of their nuclei and cell bodies selected at random from each group, adrenalin cells and noradrenalin cells were analyzed to calculate the total number of mitochondria in each adreno-medullary cell in respective zones, and the number of labeled mitochondria covered with silver grains by visual grain counting. The data were stochastically analyzed using variance and Student's t-test.

3. Results

3.1. Morphological Observations

The adreno-cortical tissues obtained from ddY strain mice at various ages from embryo day 19 to postnatal day 30, consisted of 3 layers, zona glomerulosa, zona fasciculata and zona reticularis, showed gradual development. At embryonic day 19 and postnatal day 1, the adrenocortical cells were composed mainly of polygonal cells, while the specific orientation of the 3 layers, zona glomerulosa, zona fasciculata and zona reticularis, was not yet well established. At postnatal day 3, orientation of 3 layers, zona glomerulosa, zona fasciculata and zona reticularis, became evident. At postnatal day 9 and 14, the specific structure of 3 layers was completely formed and the arrangements of the cells in respective layer became typical especially at day 14 (Figs. 1, 2, 3). Observing the ultrastructure of the adreno-cortical cells, cell organelles including mitochondria were not so well developed at perinatal and early postnatal stages from embryonic day 19 to postnatal day 3. However, these cell organelles, mitochondria,

endoplasmic reticulum, Golgi apparatus, appeared well developed from the juvenile stage at postnatal day 14 (Figs. 1, 2, 3) to the adult stages at postnatal month 1, month 2, month 6 (Figs. 4, 5, 6) and further to the senescent stage at month 12 (Figs. 7, 8, 9) and 24.

The zona glomerulosa (Figs. 1, 4, 7) of mouse adrenal cortex is the thinnest layer found at the outer zone, covered by the capsule, consisted of closely packed groups of columnar or pyramidal cells forming arcades of cell columns. The cells contained many spherical mitochondria and well developed smooth surfaced endoplasmic reticulum but a compact Golgi apparatus in day 14 (Fig. 1) to month 1, 2, 6 (Fig. 4), and to month 12 (Fig. 7) and 24 animals. The zona fasciculata was the thickest layer, consisted of polygonal cells which were larger than the glomerulosa cells, arranged in long cords disposed radially to the medulla containing many lipid droplets (Figs. 2, 5, 8). At postnatal month 1, 2 and 6, the specific structure of 3 layers was completely developed and the arrangements of the cells in respective layer became typical as adult tissues (Figs. 7, 8, 9). By high power magnification at this stage, the cytoplasmic matrix can be observed full of numerous mitochondria and considerable number of lipid droplets. Observing the ultrastructure of the adreno-cortical cells at the juvenile and adult stages, cell organelles including mitochondria were well developed from postnatal day 14 to month 1, month 2, month 6, month 12 (Figs. 7, 8, 9) and month 24. The mitochondria in the zona fasciculata (Figs. 2, 5, 8) were less numerous and were more variable in size and shape than those of the glomerulosa cells (Figs. 1, 4, 7), while the smooth surfaced endoplasmic reticulum were more developed and the Golgi apparatus was larger than the glomerulosa. In the zona reticularis (Figs. 3, 6, 9), the parallel arrangement of cell cords were anastomosed showing networks continued to the medullar cells. The mitochondria were less numerous and were more variable in size and shape than those of the glomerulosa cells like the fasciculata cells, as well as the smooth surfaced endoplasmic reticulum was developed and the Golgi apparatus was large like the fasciculata cells. Thus, the structure of the adreno-cortical cells showed changes due to development and aging at respective developmental stages.

On the other hand, The adreno-medullary tissues obtained from ddY strain mice at various ages from embryo day 19 to postnatal day 30, consisted mainly of 2 cell types, as observed by electron microscopy, adrenalin cells and noradrenalin cells, developing gradually. At embryonic day 19 and postnatal day 1, the adreno-medullary cells were composed mainly of polygonal epitheloid cells, surrounded by blood capillaries and fibroblasts [49]. The medullary cells can be divided into 2 types by the ultrastructure of granules. Some of the medullary cells possessed many granules of medium electron density which were believed to correspond to the adrenalin granules, while some other cells possessed many granules of very high electron density which were believed to correspond to the noradrenalin [49]. However, the numbers of mitochondria found in their cytoplasm were not so many. At postnatal day 1, day 3 and day 9, the 2 types of cells differentiated and the numbers of granules, both adrenalin and noradrenalin granules, increased respectively. Likewise, the numbers of mitochondria also increased from prenatal day to postnatal days. At postnatal day 14 to month 1, 2, 6, 12 and 24, the numbers of adrenalin and noradrenalin granules as well as mitochondria increased from 17-18/cell to 23-25/cell. At

postnatal month 1 and 2, the ultrastructures of 2 cell types were completely developed and the arrangements of the cells in the medulla became typical as adult tissues. The ultrastructure of 2 cell types appeared almost the same as in the previous study [49]. Thus, the ultrastructure of the adrenal medullary cells did not show any changes due to aging at respective senescent stages at postnatal month 12 (Fig. 1) and month 24 (Fig. 2). The all data from embryonic day 19 to postnatal day 1, 3, 9, 14, month 1, 2, 6, 12 to 24, were stochastically analyzed using variance and Student's t-test. The increases of mitochondrial numbers in both adrenalin and noradrenalin cells from embryonic day 19 to postnatal month 6 were considered to be significant at P value <0.01.

Fig. 1. Electron microscopic radioautogram of the zona glomerulosa of a juvenile mouse at postnatal day 14, labeled with ^3H -uridine showing RNA synthesis (several silver grains) in the nucleus as well as in a few mitochondria. x 3,000.

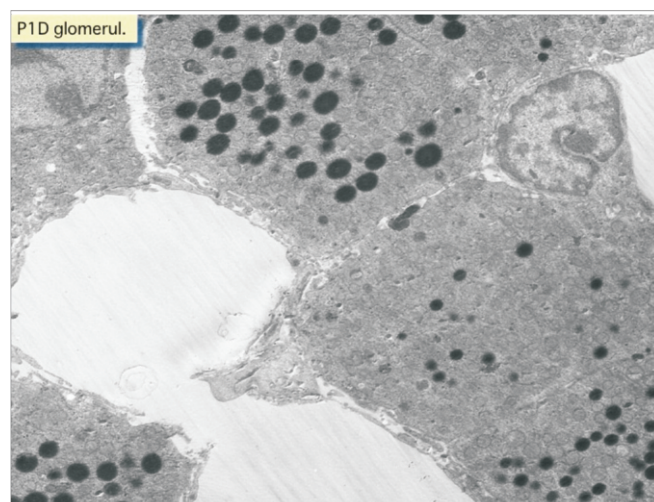


Fig. 2. Electron microscopic radioautogram of the zona fasciculata of a juvenile mouse at postnatal day 14, labeled with ^3H -uridine showing RNA synthesis (several silver grains) in the nucleus as well as in a few mitochondria. x 3,000.

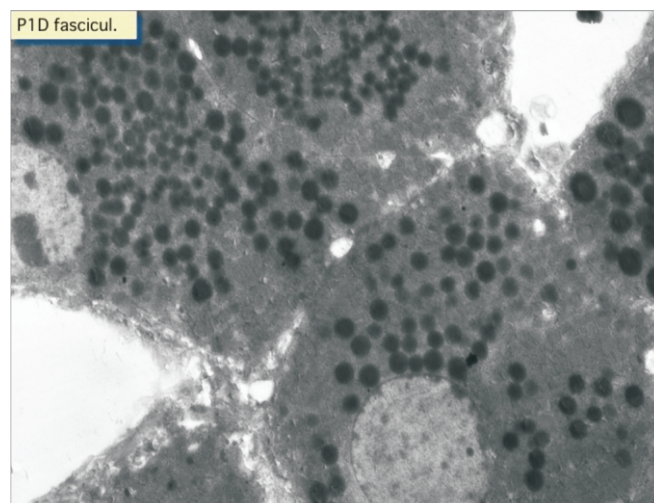


Fig. 3. Electron microscopic radioautogram of the zona reticularis of a juvenile mouse at postnatal day 14, labeled with ^3H -uridine showing RNA synthesis (several silver grains) in the nucleus as well as in a few mitochondria. x3,000.

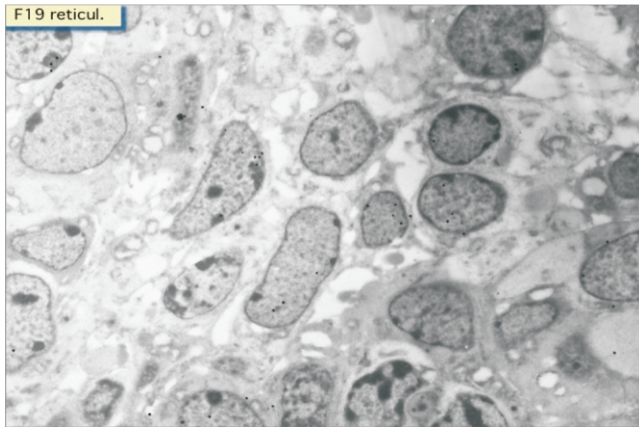


Fig. 4. Electron microscopic radioautogram of the zona glomerulosa of a mature adult mouse aged at postnatal month 2, labeled with ^3H -uridine showing RNA synthesis (several silver grains) in the nucleus as well as in several mitochondria. x3,000.

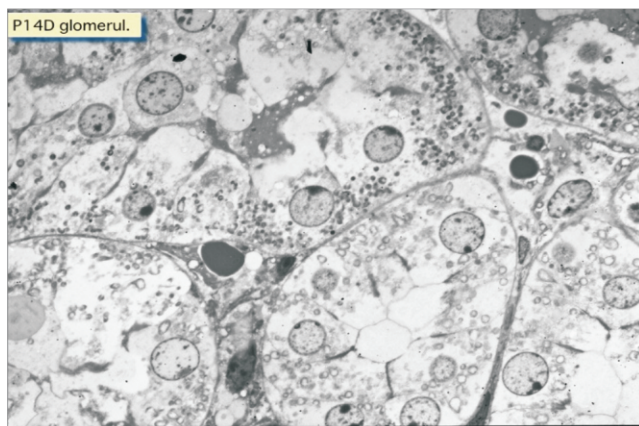


Fig. 5. Electron microscopic radioautogram of the zona fasciculata of a mature adult mouse aged at postnatal month 2, labeled with ^3H -uridine showing RNA synthesis (several silver grains) in the nucleus as well as in a few mitochondria. x3,000.

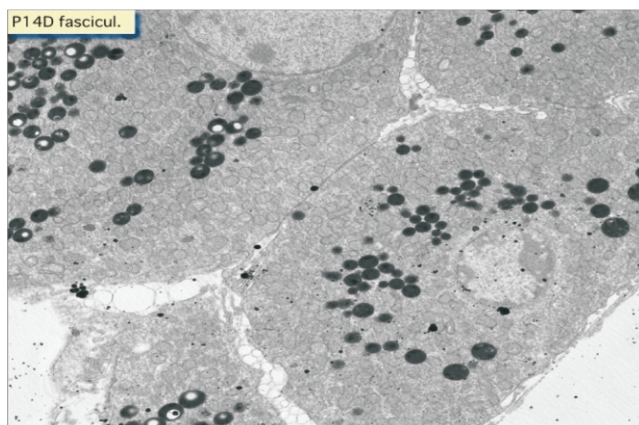


Fig. 6. Electron microscopic radioautogram of the zona reticularis of a mature adult mouse aged at postnatal month 2, labeled with ^3H -uridine showing RNA synthesis in the nucleus as well as in a few mitochondria. x3,000.

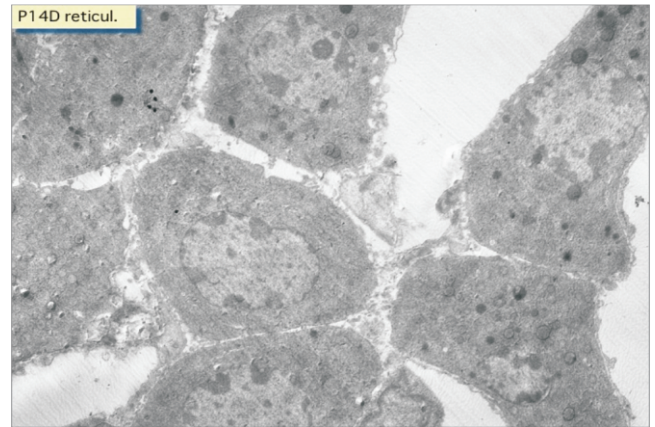


Fig. 7. Electron microscopic radioautogram of the zona glomerulosa of an old adult mouse aged at postnatal month 12, labeled with ^3H -uridine showing RNA synthesis (few silver grains) in the nucleus as well as in a few mitochondria. x3,000.

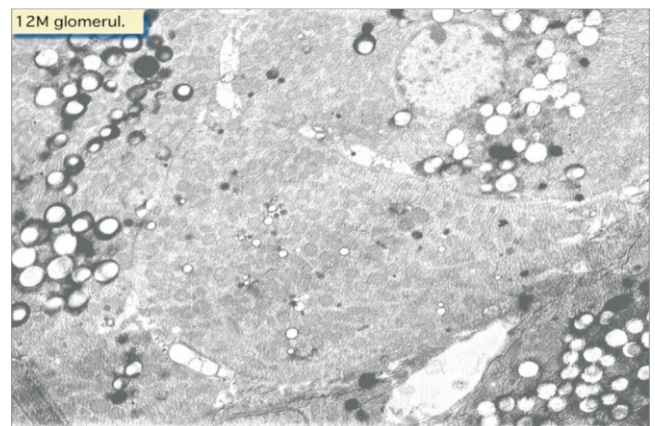


Fig. 8. Electron microscopic radioautogram of the zona fasciculata of an old adult mouse aged at postnatal month 12, labeled with ^3H -uridine showing RNA synthesis (few silver grains) in the nucleus and in a few mitochondria. x3,000.

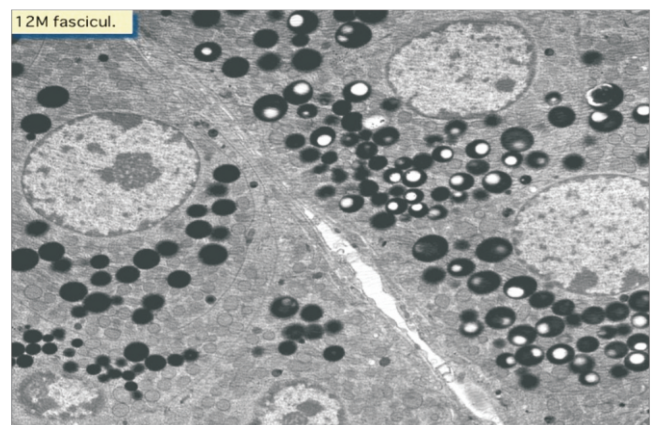


Fig. 9. Electron microscopic radioautogram of the zona reticularis of an old adult mouse aged at postnatal month 12, labeled with ^3H uridine showing RNA synthesis in the nucleus and a few mitochondria. x3,000.

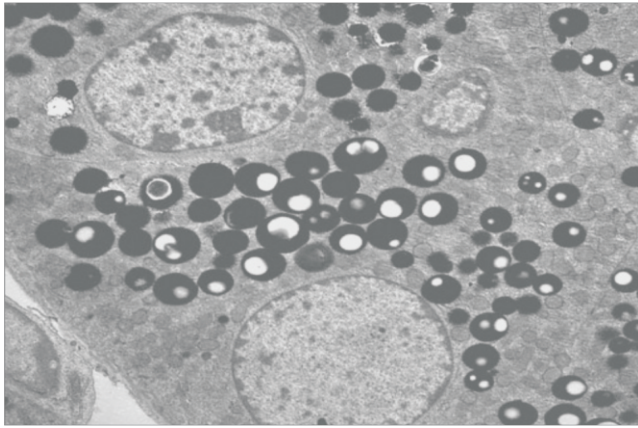


Fig. 10. Electron microscopic radioautogram of the adreno-medullary tissues of a postnatal adult mouse at month 2, labeled with ^3H uridine, showing RNA synthesis (several silver grains) in the nuclei as well as in a few mitochondria of 2 adrenalin cells at bottom right and a noradrenalin cell at left center. x3,000.

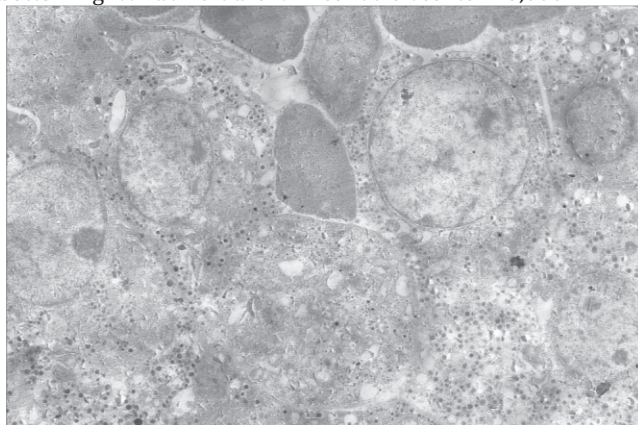


Fig. 11. Electron microscopic radioautogram of the adreno-medullary tissues of a postnatal senescent mouse at month 12 (year 1), labeled with ^3H uridine, showing RNA synthesis (several silver grains) in the cytoplasm especially over several mitochondria of an adrenalin cell at left and a noradrenalin cell at

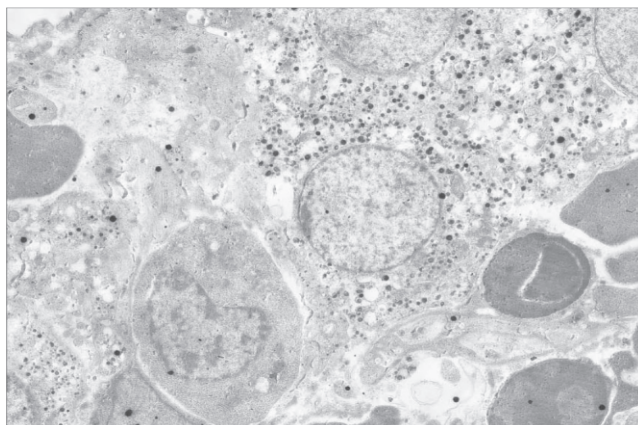


Fig. 12. Electron microscopic radioautogram of the adreno-medullary tissues of a postnatal senescent mouse at year 2, labeled with ^3H uridine, showing RNA synthesis (several silver grains) in the cytoplasm especially over several mitochondria of 2 adrenalin cells at upper center and left and a noradrenalin cell at bottom right. x3,000.

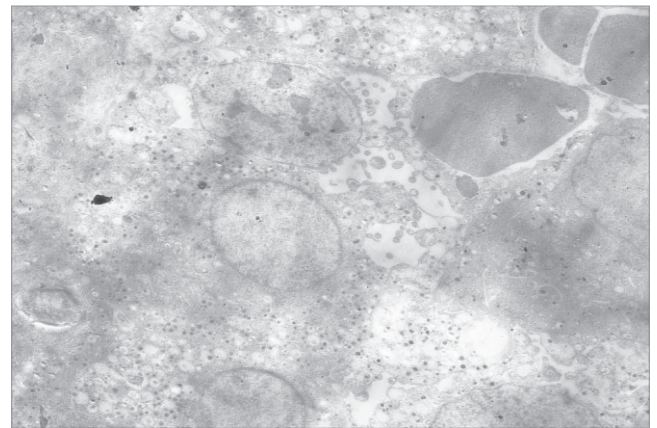


Fig. 13. Histogram showing aging changes of the average numbers of mitochondria per cell in each adreno-cortical cell in the 3 layers of respective animals in 10 aging groups.

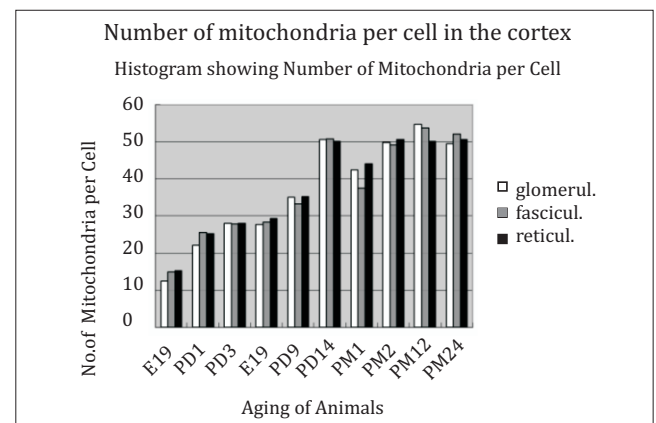


Fig. 14. Histogram showing aging changes of the average numbers of labeled mitochondria with ^3H uridine showing RNA synthesis per cell in each adreno-cortical cell in the 3 layers of respective animals in 10 aging groups.

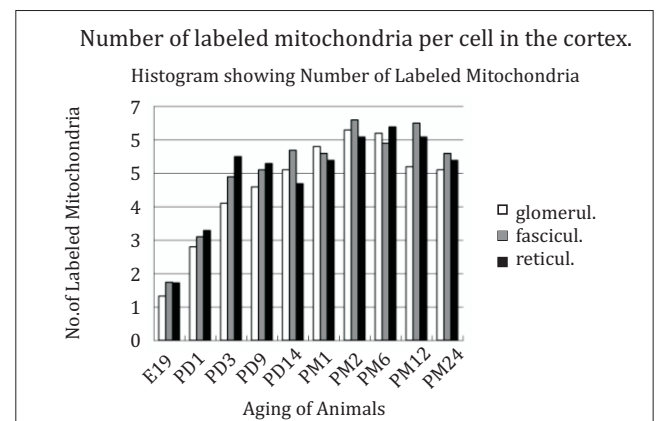


Fig. 15. Histogram showing aging changes of the average labeling index of mitochondria labeled with ^3H uridine showing RNA synthesis per cell in each adreno-cortical cell in the 3 layers of respective animals in 10 aging groups.

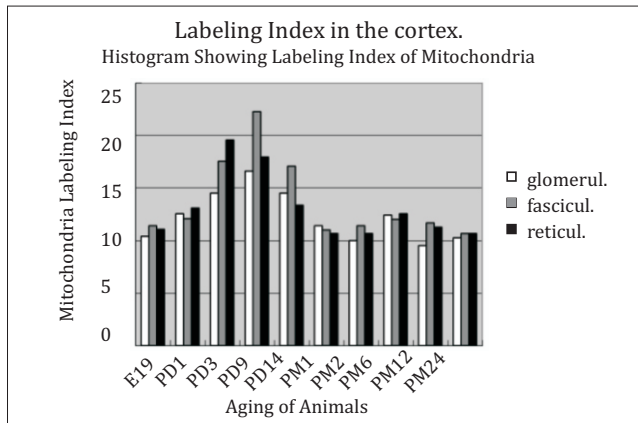
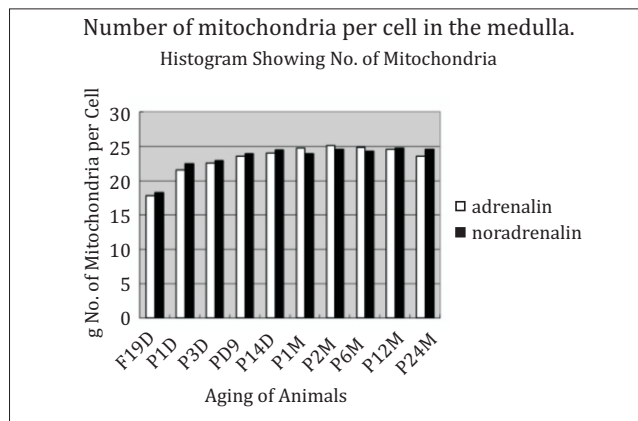


Fig. 16. Histogram showing the number of mitochondria per each adreno-medullary cell, either adrenalin cell or noradrenalin cell of respective animals in 10 groups during the development and aging from fetal day 19 to postnatal month 24. The number of mitochondria per cell increased from perinatal stage to postnatal day 14 and to postnatal month 1, kept the maxima to month 2, 6, 12 and 24 (year 2).



3.2. Radioautographic Observations

Observing EM radioautograms, the silver grains were found over the nuclei of some adreno-cortical cells labeled with ^3H -uridine, demonstrating RNA synthesis in all aging stages from perinatal stages at embryonic day 19, postnatal day 1 and day 3, day 9, and day 14 (Figs. 1, 2, 3) and adults at month 1, month 2, month 6 (Figs. 4, 5, 6), month 12 (Figs. 7, 8, 9) and month 24. Those labeled cells were found in all the 3 layers, the zona glomerulosa (Figs. 1, 4, 7), the zona fasciculata (Figs. 2, 5, 8) and the zona reticularis (Figs. 3, 6, 9), at respective aging stages. In the labeled adreno-cortical cells in 3 layers the silver grains were mainly localized over the euchromatin of the nuclei and nucleoli, or a few or several silver grains were found over cytoplasmic organelles such as endoplasmic reticulum, ribosomes and mitochondria showing RNA synthesis incorporating ^3H -uridine. The localizations of silver grains over the mitochondria were mainly on the mitochondrial matrices and some over the mitochondrial membranes when observed by high power magnification.

Fig. 17. Histogram showing the number of labeled mitochondria per each adreno-medullary cell either adrenalin cell or noradrenalin cell of respective animals in 10 groups during the development and aging from fetal day 19 to postnatal month 24. The number of labeled mitochondria per cell increased from perinatal stage to juvenile adult stage at postnatal month 1, then kept the maxima from month 6 to month 24 (year 2).

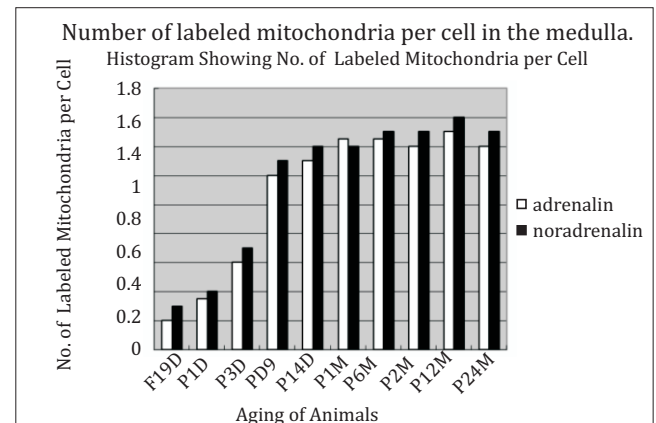
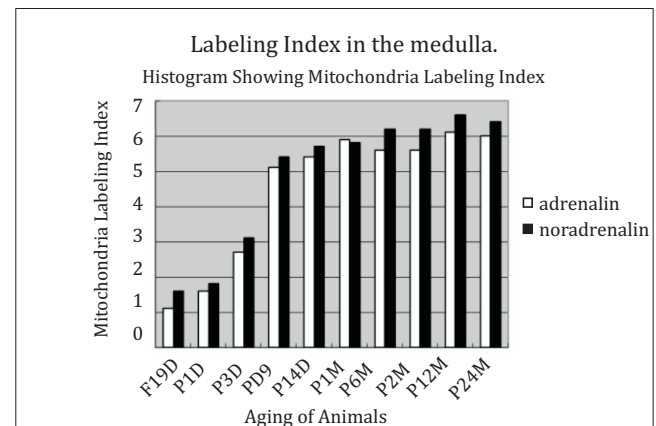


Fig. 18. Histogram showing the labeling index of mitochondria per each adreno-medullary cell in either adrenalin cell or noradrenalin cell of respective animals in 10 groups during the development and aging from fetal day 19 to postnatal month 24. The labeling index of mitochondria increased from perinatal stage to juvenile adult stage at postnatal month 1, then kept the maxima from month 6 to month 24 (year 2).



On the other hand, observing electron microscopic radioautograms of adreno-medullary cells, the silver grains were found over the nuclei of some adreno-medullary cells labeled with ^3H -uridine, demonstrating RNA synthesis mainly in perinatal stages at embryonic day 19, postnatal day 1 and day 3, increased to day 9, day 14, and month 1, reaching the maximum showing labeled mitochondria around 1.4-1.5/cell, then kept the maximum to adults at month 2, 6, 12 and 24, showing labeled mitochondria around 1.3-1.5/cell.

The labeled cells were found in all the 2 cell types, adrenalin cells (Figs. 10, 11) and noradrenalin cells (Figs. 10, 11), at respective aging stages from embryo day 19 (Fig. 10) to postnatal day 1, 3, 9, 14 (Fig. 11), month 1, 2, 6, 12 and 24. In the labeled adreno-medullary cells the silver grains were localized over the euchromatin of the nuclei as well as over the mitochondria showing RNA synthesis incorporating ^3H -uridine.

3.3. Quantitative Analysis

3.3.1. Number of mitochondria per cell

Preliminary quantitative analysis on the number of mitochondria in 10 adreno-cortical cells whose nuclei and cytoplasm were labeled with silver grains and other 10 cells whose nuclei and cytoplasm were not labeled in each aging group revealed that there was no significant difference between the number of mitochondria and the labeling indices ($P < 0.01$). Thus, the number of mitochondria and the labeling indices were calculated regardless whether their nuclei were labeled or not. The results obtained from the number of mitochondria in adreno-cortical cells in the 3 layers of respective animals in 10 aging groups at perinatal stages, prenatal embryo day 19, postnatal day 1, 3, 9 and 14, showed an gradual increase from the prenatal day 19 (glomerulosa 12.5, fasciculata 14.5, reticularis 15.2/cell) to postnatal day 14 (glomerulosa 35.1, fasciculata 33.2, reticularis 35.3/cell), and to adult stages at postnatal month 1 (glomerulosa 50.7, fasciculata 50.8, reticularis 49.2/cell), then slightly decreased at month 2 (glomerulosa 42.4, fasciculata 37.6, reticularis 44.1/cell), but kept plateau from month 6 (glomerulosa 49.8, fasciculata 49.2, reticularis 50.6/cell), to month 12 (glomerulosa 54.7, fasciculata 53.8, reticularis 50.2/cell) and month 24 (glomerulosa 49.5, fasciculata 52.1, reticularis 50.6/cell), as is shown in Fig. 13. The increase from embryo day 19 to postnatal month 1 was stochastically significant ($P < 0.01$).

On the other hand, the numbers of mitochondria and the labeling indices were calculated. The results obtained from the numbers of mitochondria in adreno-medullary cells of respective animals in 10 aging groups from perinatal stages, embryo day 19 to postnatal day 1, 3, 9, 14, and month 1, 2, 6, 12 and 24 showed an gradual increase from the prenatal day 19 (adrenalin 17.8, noradrenalin 18.2/cell) to postnatal day 1, day 3, day 9, day 14 (adrenalin 24.0, noradrenalin 24.4/cell), and to adult stages at postnatal month 1 (adrenalin 24.7, noradrenalin 24.5/cell), month 2 (adrenalin 25.1, noradrenalin 24.3/cell) and month 6 (adrenalin 24.8, noradrenalin 24.3/cell), 12 and 24 (adrenalin 23.5, noradrenalin 24.5/cell) as is shown in Fig. 16. The increase of mitochondrial numbers in both adrenalin and noradrenalin cells from embryonic day 19 to postnatal month 1, 2, 6, 12 and 24 was considered to be significant at P value < 0.01 . However, the slight difference from month 1 to month 24 was not significant.

3.3.2 Mitochondrial RNA synthesis

The results of visual grain counting on the number of mitochondria labeled with silver grains obtained from 10 adreno-cortical cells in the 3 layers of each animal labeled with ^3H -uridine demonstrating RNA synthesis in 10 aging groups at perinatal stages, prenatal embryo day 19, postnatal day 1, 3, 9 and 14, month 1, 3, 6, 12 and 24, are plotted in Fig. 11. The results demonstrated that the numbers of labeled mitochondria with ^3H -uridine showing RNA synthesis per cell gradually increased from prenatal embryo day 19 (glomerulosa 1.3, fasciculata 1.7, reticularis 1.7/cell) to postnatal day 1 (glomerulosa 2.8, fasciculata 3.1, reticularis 3.3/cell), day 3 (glomerulosa 4.1, fasciculata 4.9, reticularis 5.5/cell), day 9 (glomerulosa 4.6, fasciculata 5.1, reticularis 5.3/cell), day 14 (glomerulosa 5.1, fasciculata 5.7, reticularis 4.7/cell), and month 1 (glomerulosa 5.8, fasciculata 5.6, reticularis 5.4/cell) and month 2 (glomerulosa 6.3, fasciculata 6.6, reticularis 6.1/cell), reaching the maximum, then decreased to month 6 (glomerulosa 6.2, fasciculata 5.9, reticularis 6.4/cell), month 12 (glomerulosa 5.2, fasciculata 6.5,

reticularis 6.1/cell) and 24 (glomerulosa 5.1, fasciculata 5.6, reticularis 5.4/cell) as is shown in the histogram (Fig. 14).

On the other hand, the number of mitochondria labeled with silver grains obtained from 10 adreno-medullary cells of each animal labeled with ^3H -uridine demonstrating RNA synthesis in 10 aging groups at perinatal stages, to postnatal day 1, 3, 9 and 14, month 1, 3, 6, 12 and 24, were counted. The results demonstrated that the numbers of labeled mitochondria with ^3H -uridine per cell gradually increased from prenatal embryo day 19 (adrenalin 0.2, noradrenalin 0.3/cell), to postnatal day 1 (adrenalin 0.35, noradrenalin 0.4/cell), day 3 (adrenalin 0.6, noradrenalin 0.7/cell), day 9 (adrenalin 1.2, noradrenalin 1.3/cell), day 14 (adrenalin 1.3, noradrenalin 1.4/cell), to month 1 (adrenalin 1.45, noradrenalin 1.5/cell), month 2 (adrenalin 1.45, noradrenalin 1.5/cell), month 6 (adrenalin 1.4, noradrenalin 1.5/cell), month 12 (adrenalin 1.5, noradrenalin 1.6/cell) and 24 (adrenalin 1.4, noradrenalin 1.5/cell). The data were stochastically analyzed using variance and Student's t -test. The increases of the numbers of labeled mitochondria in both adrenalin and noradrenalin cells from embryo day 19 to postnatal month 1 was stochastically significant ($P < 0.01$). However, the slight difference between month 1 to 24 was not significant.

3.3.3. The labeling index

From the above results, the labeling indices of adreno-cortical cells in respective aging stages were calculated from the number of labeled mitochondria (Fig. 14) dividing by the number of total mitochondria per cell (Fig. 13), which were plotted in Fig. 15, respectively.

The results showed that the labeling indices gradually increased from prenatal day 19 (glomerulosa 10.4, fasciculata 11.4, reticularis 11.1%) to postnatal newborn stage at postnatal day 1 (glomerulosa 12.6, fasciculata 12.1, reticularis 13.1%) and day 3 (glomerulosa 14.5, fasciculata 17.6, reticularis 19.6%), and to juvenile stage at postnatal day 9 (glomerulosa 16.6, fasciculata 22.3, reticularis 18.0%), reaching the maximum, and decreased to day 14 (glomerulosa 14.5, fasciculata 17.1, reticularis 13.4%) and to the adult stage at month 1 (glomerulosa 11.4, fasciculata 11.0, reticularis 10.7%) and month 2 (glomerulosa 10.0, fasciculata 11.4, reticularis 10.7%), to month 6 (glomerulosa 12.4, fasciculata 12.0, reticularis 12.6%) to month 12 (glomerulosa 9.5, fasciculata 11.7, reticularis 11.3%) and finally to senescence at month 24 (glomerulosa 10.3, fasciculata 10.7, reticularis 10.7%), as is shown in the histogram (Fig. 15).

On the other hand, the labeling indices of mitochondrial RNA synthesis in 2 cell types in respective aging stages were calculated from the number of labeled mitochondria (Fig. 17) dividing by the number of total mitochondria per cell (Fig. 16) which were plotted in Fig. 18, respectively.

The results showed that the labeling indices gradually increased from prenatal day 19 (adrenalin 1.1, noradrenalin 1.6%) to postnatal newborn day 1 (adrenalin 1.6, noradrenalin 1.8%), day 3 (adrenalin 2.7, noradrenalin 3.1%), day 9 (adrenalin 5.1, noradrenalin 5.4%) to juvenile stage at day 14 (adrenalin 5.4, noradrenalin 5.7%), to adult stages at month 1 (adrenalin 5.9, noradrenalin 5.8%), month 2 (adrenalin 5.6, noradrenalin 6.2%), month 6 (adrenalin 5.6, noradrenalin 6.2%), month 12 (adrenalin 6.1, noradrenalin 6.6%) and 24 (adrenalin 6.0, noradrenalin 6.4%) as shown in Fig. 5. From the results, the increases of the mitochondrial labeling indices in both adrenalin and noradrenalin cells from embryo day 19 to postnatal month 1, was stochastically significant ($P < 0.01$). On the other hand, the difference between month 1 to 24 was not significant.

4. Discussion

From the results obtained at present, it was shown that intramitochondrial RNA synthesis was observed in adreno-cortical cells in the 3 layers, zona glomerulosa, zona fasciculata and zona reticularis, of aging and senescent mice at various ages from prenatal embryos to postnatal newborn, juvenile and young adult to senescent stages until postnatal month 24 (year 2) and the number of mitochondria per cell showed increases due to aging, while the number of labeled mitochondria per cell and the labeling indices showed increases, reaching the maxima at postnatal day 9, and decreases due to aging and senescence. To the contrary, it was also shown that the increases of the mitochondrial labeling indices in both adrenalin and noradrenalin cells of the adreno-medullary cells from embryo day 19 to postnatal month 1, was stochastically significant ($P < 0.01$), while the difference between month 1 to 24 was not significant.

As for the macromolecular synthesis in various cells in various organs of experimental animals observed by light and electron microscopic radioautography, it is well known that the silver grains due to radiolabeled 3H-thymidine demonstrate DNA synthesis [1, 2, 3, 48, 49], while the grains due to 3H-uridine demonstrate RNA synthesis [22, 25, 26, 28].

The previous results obtained from the studies on DNA synthesis of the adreno-cortical cells of aging mice by light microscopic radioautography revealed that silver grains indicating DNA synthesis incorporating 3H-thymidine were observed over the nuclei of some adreno-cortical cells at perinatal stages from postnatal day 1 to 14 [39, 40]. However, they did not observe the intramitochondrial DNA synthesis. In the previous study [41], the numbers of silver grains showing nuclear DNA synthesis, as expressed by grain counting, did not give any significant difference between the cells in the 3 layers in the same aging groups. These results indicated that the amount of DNA synthesized in one nucleus was almost the same as in any other cells independent upon whether the nucleus belonged to any layers of the adrenal cortex. However, these differences between the 3 layers at respective aging groups were not stochastically significant ($P < 0.01$). These results indicated that the DNA synthetic activity in the nuclei of 3 layers of the adrenal cortex did not show any difference. To the contrary, the numbers of mitochondria labeled with 3H-thymidine per cell as well as the labeling indices between the aging groups increased gradually from prenatal embryo day 19, to postnatal day 1, 3, 9 and 14, to postnatal month 1 and 2, then decreased to month 6, 12 and 24. These decreases were stochastically significant ($P < 0.01$). These results indicated that the mitochondria in adreno-cortical cells proliferated from perinatal and postnatal newborn stage to adult stage at postnatal month 1 and 2, reaching the maxima, then lost their proliferating activities from aged stage at month 6 to senescent stage up to month 24.

To the contrary, the radioautograms in the present study showing incorporations of 3H-uridine into mitochondria indicating mitochondrial RNA synthesis resulted in silver grain localization over the mitochondria independently from the nuclei whether the nuclei were labeled with silver grains or not in almost all the cells in the 3 layers of the adreno-cortical and adreno-medullary cells from prenatal embryo day 19 to postnatal day 1, 3, 9 and 14, to postnatal month 1, 2, 6, 12 and 24, during the development and aging. The numbers of labeled mitochondria showing RNA synthesis increased from perinatal day to postnatal adult stage at month 2, then kept plateau, while the labeled

mitochondria with 3H-uridine showing RNA synthesis increased from perinatal stage to postnatal adult stage at month 2, then decreased at month 24, while the labeling indices increased from perinatal embryonic day to postnatal newborn and juvenile stages at day 9, then decreased from day 14 to senescence at month 24, then decreased to the adult stages at month 1 and 2, to month 6, 12 and 24. These changes demonstrate the respective aging changes. The results obtained previously [41] indicated that mitochondria in the adreno-cortical and adreno-medullary cells proliferated from newborn to adult stages around month 1 and 2, showing mitochondrial DNA synthesis, while the present results indicate that mitochondrial RNA synthesis increased from newborn stage to postnatal day 9, then decreased from day 14, reaching the maxima, then decreased to month 24, but the RNA synthetic activity was kept from day 14 to month 12 which decreased to senescent at month 24 due to aging.

With regard to the DNA in mitochondria in animal cells or plastids in plant cells, many studies have been previously reported in various cells of various plants and animals since 1960s [52, 53, 54, 55]. Most of these authors observed DNA fibrils in mitochondria which were histochemically extracted by DNase. Electron microscopic observation of the DNA molecules isolated from the mitochondria revealed that they were circular in shape, with a circumference of 5-6 μm [56]. It was calculated that such a single molecule had a molecular weight of about 107 daltons [57]. Mitochondria of various cells also contained DNA polymerase, which was supposed to function in the replication of the mitochondrial DNA [58]. On the other hand, the incorporations of ^3H -thymidine into mitochondria demonstrating DNA synthesis were observed by means of electron microscopic radioautography in lower organism such as slime mold [59, 60], tetrahymena [61] or chicken fibroblasts in tissue culture under abnormal conditions [62]. However, these authors used old-fashioned developers consisting of methol and hydroquinone (MQ-developer), which produced coarse spiral silver grains resulting in inaccurate localization over cell organelles when observed by electron microscopy. All of these authors showed photographs of electron radioautographs with large spiral-formed silver grains (2-3 μm in diameter) localizing not only over the mitochondria but also outside the mitochondria. In order to obtain smaller silver grains, we first used elon-ascorbic acid developer after gold latensification [1, 2, 3, 4, 5, 6], which produced comma-shaped smaller silver grains (0.4-0.8 μm in diameter), then later we used phenidon developer after gold latensification, producing dot-like smaller silver grains (0.2-0.4 μm in diameter) localizing only inside the mitochondria showing ultrahigh resolution of radioautograms [30, 48, 49, 50, 51]. These papers were the first which demonstrated intramitochondrial DNA synthesis incorporating ^3H -thymidine with accurate intramitochondrial localization in avian and mammalian cells. With regard to the resolution of electron microscopic radioautography, on the other hand, many authors discussed the size of silver grains under various conditions and calculated various values of resolutions [4, 5, 63, 64, 65]. Those authors who used the M-Q developers maintained the resolution to be 100-160 nm [63, 64], while those authors who used the elon-ascorbic acid developer [4, 5, 65] calculated it to be 25-50 nm. When we used phenidon developer at 16°C for 1 min after gold latensification, we could produce very fine dot-shaped silver grains and obtained the resolution around 25 nm as formerly reported [30, 48, 49, 50, 51, 66]. For the analysis of electron radioautographs, Salpeter et al. [63] proposed to use the half-distance and very complicated calculations through which respective coarse spiral-shaped silver

grains were judged to be attributable to the radioactive source in a certain territory within a resolution boundary circle. However, since we used phenidon developer after gold latensification to produce very fine dot-shaped silver grains, we judged only the silver grains which were located in the mitochondria which were dot-shaped very fine ones to be attributable to the mitochondria without any problem as was formerly discussed [3 - 5, 48 - 51].

Then we also demonstrated intramitochondrial DNA synthesis incorporating ^3H -thymidine in some other established cell lines originated from human being such as HeLa cells [3, 4, 5, 6] or mitochondrial fractions prepared from *in vivo* mammalian cells such as rat and mouse [7, 8, 9]. It was later commonly found in various cells and tissues not only *in vitro* obtained from various organs *in vivo* such as the cultured human HeLa cells [13, 67], cultured rat sarcoma cells [12], mouse liver and pancreas cells *in vitro* [11, 14, 31], but also *in vivo* cells obtained from various organs such as the salivary glands [15], the liver [16 - 29], the pancreas [30, 31], the trachea [32], the lung [33], the kidney [34], the testis [35, 36], the uterus [37, 38], the adrenal gland [39, 40, 41], the brain [42], and the retina [43 - 47] of mice, rats and chickens. Thus, it is clear that all the cells in various organs of various animals synthesize DNA not only in their nuclei but also in their mitochondria.

The relationship between the intramitochondrial DNA synthesis and cell cycle was formerly studied in synchronized cells and it was clarified that the intramitochondrial DNA synthesis was performed without nuclear involvement [4]. However, the relationship between the DNA synthesis and the aging of individual animals and men has not yet been clarified except a few papers recently published by Korr and associates on mouse brain [69-72]. They reported both nuclear DNA repair, measured as nuclear unscheduled DNA synthesis, and cytoplasmic DNA synthesis labeled with ^3H -thymidine in several types of cells in brains such as pyramidal cells, Purkinje cells, granular cells, glial cells, endothelial cells, ependymal cells, epithelial cells as observed by light microscopic radioautography using paraffin sections. They observed silver grains over cytoplasm of these cells by light microscopy and maintained that it was reasonable to interpret these labeling as ^3H -DNA outside the nuclei, which theoretically belonged to mitochondrial DNA without observing the mitochondria by electron microscopy. From the results, they concluded that distinct types of neuronal cells showed a decline of both unscheduled DNA and mitochondrial DNA syntheses with age in contrast that other cell types, glial and endothelial cells, did not show such age-related changes without counting the number of mitochondria in respective cells nor counting the labeling indices at respective aging stages. Thus, their results from the statistics obtained from the cytoplasmic grain counting seems to be not accurate without observing mitochondria directly. To the contrary, we had studied DNA synthesis in the livers of aging mice [16-29] and clearly demonstrated that the number of mitochondria in each hepatocytes, especially mononucleate hepatocytes, increased with the ages of animals from the perinatal stages to adult and senescent stages, while the numbers of labeled mitochondria and the labeling indices increased from the perinatal stages, reaching a maximum at postnatal day 14, then decreased. Our previous studies [22, 23] also clarified that the DNA synthesis and cell proliferation by mitosis were the most active in the nuclei of mononucleate hepatocytes at the perinatal stages in contrast that binucleate cells were less active at the perinatal stage but the

number of binucleate hepatocytes increased at senescent stages and the results suggest the possibility that the mitochondria in mononucleate hepatocytes synthesized their DNA by themselves which peaked at postnatal day 14 in accordance with the proliferation of mononucleate hepatocytes while binucleate hepatocytes increased after the perinatal stage and did not divide but remained binucleate keeping many mitochondria in their cytoplasm which were more in number than mononucleate hepatocytes at the senescent stage.

Thus, our previous papers were the first which dealt with the relationship between the DNA synthesis and aging in hepatocytes of mice *in vivo* at various ages by means of electron microscopic radioautography observing the small dot-like silver grains, due to incorporations of ^3H -thymidine, which exactly localized inside the mitochondria. Our previous results also revealed that an increase was observed by direct observation on mitochondria at electron microscopic level and obtaining accurate mitochondrial numbers and labeling indices in adreno-cortical cells in 8 groups of developing mice. There was a discrepancy between our results from the hepatocytes [22, 23] as well as the adreno-cortical cells [39, 40, 41] and the results from the several types of cells in the brains by Korr et al. [69-72]. The reason for this difference might be due to the difference between the cell types (hepatocytes or adrenal cells and the brain cells) or the difference between the observation by electron microscopy, i.e., direct observation of mitochondria in our results or light microscopy, i. e., indirect observation of mitochondria without observing any mitochondria directly and misinterpretation by Korr et al. [69-72].

The results obtained from the adrenal glands of aging mice at present should form a part of special cytochemistry [48], as well as a part of special radioautography [49], i.e., the application of radioautography to the adrenal glands, as was recently reviewed by the present author. We expect that such special radioautography and special cytochemistry should be further developed in all the organs of animals in the future.

5. Conclusions

From the results obtained at present, it was concluded that almost all the cells in the 3 layers of the adrenal cortex of mice at various ages, from prenatal embryo day 19 to postnatal newborn, day 1, 3, 9 and 14, and to postnatal month 1, 2, 6, 12 and 24, were labeled with silver grains showing RNA synthesis with ^3H -uridine in their mitochondria. Quantitative analysis on the number of mitochondria in adreno-cortical cells in the 3 layers resulted in an increase from the prenatal day to postnatal day 1, 3, 9, 14, and month 1 and 2, and 6, reaching the maximum at postnatal month 12, then a little decreased to month 24. To the contrary, the numbers of labeled mitochondria with ^3H -uridine showing RNA synthesis increased from perinatal stage to postnatal juvenile stage at day 9, and decreased to aging and senescence, while the mitochondrial labeling index also increased from prenatal day to postnatal day 1, 3 and 9, reaching the maximum at postnatal day 9, then decreased to day 14, month 1, 2, 6, 12 and 24. To the contrary, it was shown that the increases of the mitochondrial labeling indices in both adrenalin and noradrenalin cells of the adreno-medullary cells from embryo day 19 to postnatal month 1, was stochastically significant ($P < 0.01$) but the difference between month 1 to 24 was not significant.

These results demonstrate that the numbers of mitochondria in adreno-cortical cells increased by proliferating themselves synthesizing mitochondrial DNA at perinatal stages to postnatal

month 2 due to aging of animals, while the activity of mitochondrial RNA synthesis in the adreno-cortical cells and adreno-medullary cells in developing and senescent mice changed, increased to month 2 at adult stage and decreased to month 6 to 24 at senescence, due to aging of individual animals.

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