LIGHT AND ELECTRON MICROSCOPIC ANALYSIS OF THE NORMAL AND TRANSPLANTED PITUITARY OF THE MEXICAN AXOLOTL AMBYSTOMA MEXICANUM

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY
THOMAS G. CONNELLY

1970

This is to certify that the

thesis entitled

LIGHT AND ELECTRON MICROSCOPIC ANALYSIS
OF THE NORMAL AND TRANSPLANTED PITUITARY
OF THE MEXICAN AXOLOTL AMBYSTOMA MEXICANUM

presented by

Thomas G. Connelly

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Zoology

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Major professor

Date June 22, 1970

O-169

ABSTRACT

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Interest in the ultrastructure of the pituitary of the Mexican axolotl has been generated by the animal's inability to metamorphose spontaneously (neotony) and the ability of its pituitary gland to support survival in hypophysectomized newts (Tazawa, 1964b). Since this survival enhancement is comparable to that exhibited when hypophysectomized newts receive pituitary homografts or are treated with prolactin and thyroxine, it had hoped that an analysis of the normal and ectopically transplanted axolotl pituitary would give some information regarding the functional activity (prolactin and TSH) of some of the cells in this gland. Therefore, light and electron microscopic observations were made on axolotl pituitaries which had been transplanted to the lower jaws of hypophysectomized adult newts for one week, three weeks and four months. In addition, autografted and homografted axolotl pituitaries were observed at one and three weeks after transplantation. Newt pituitaries which had been transplanted to hypophysectomized axolotls were also observed. Finally, pituitaries of two-year old sexually mature axolotls and axolotls which had been treated with thyroxine to induce metamorphosis were also fixed and examined. Normal untreated pituitaries served as

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Interest in the ultrastructure of the pituitary of the Mexican axolotl has been generated by the animal's inability to metamorphose spontaneously (neoteny) and the ability of its pituitary gland to support survival in hypophysectomized newts (Tassava, 1969b). Since this survival enhancement is comparable to that obtained when hypophysectomized newts receive pituitary autografts or are treated with prolactin and thyroxine, it was hoped that an analysis of the normal and ectopically transplanted axolotl pituitary would give some information regarding the functional activity (prolactin and TSH) of some of the cells in this gland. Therefore, light and electron microscopic observations were made on axolotl pituitaries which had been transplanted to the lower jaws of hypophysectomized adult newts for one week, three weeks and four months. In addition, autografted and homografted axolotl pituitaries were observed at one and three weeks after transplantation. Newt pituitaries which had been transplanted to hypophysectomized axolotls were also observed. Finally, pituitaries of two-year old sexually mature axolotls and axolotls which had been treated with thyroxine to induce metamorphosis were also fixed and examined. Normal untreated pituitaries served as

controls for all experiments.

The pituitary of a young Mexican axolotl is composed of three distinct regions, the pars nervosa, the pars intermedia and the pars distalis. The cellular characteristics of each region are: Pars nervosa-nerve fibers and electron dense neurosecretory granules; pars intermedia - vesicles 200-220 mμ in diameter, oval cells; pars distalis - five cell types, I with granules 260-330 mμ of variable electron density, II with granules 330-520 mμ diameter, variable electron density, vesicles 130-330 mμ in diameter and globules 1μ in diameter, III with granules 330 mμ in diameter uniformly electron dense, IV with vesicles 200-220 mμ in diameter, V are triangular cells having long cytoplasmic processes containing microfibrils. In addition, many cells of the pituitary contain large membrane-enclosed multitubular structures which take a variety of shapes.

After ectopic auto-, homo-, or xenoplastic transplantation, large amounts of rough-surfaced endoplasmic reticulum are found in the pars intermedia cells. This corresponds to a marked skin darkening in the host probably caused by hypersecretion of melanocyte stimulating hormone after release of the pars intermedia from hypothalamic control.

By three weeks after xenoplastic transplantation, identification of the different cell types within the pars distalis becomes difficult. By four months after grafting, almost all granulation is lost from pituitary

cells, with the type III cells the most often recognizable type. Large amounts of rough-surfaced endoplasmic reticulum are present in most of the graft cells. The retention of some granulation by type III cells implicates them as an acidophilic cell type and thus as a possible source of prolactin in the axolotl pituitary (see van Oordt, 1968; Pasteels, 1967; Doerr-Schott, 1968; Masur, 1969). At three weeks after transplantation, all cell types are easily visible in auto- or homografted pituitaries.

Reciprocally transplanted newt pituitaries (newt to axolotl) are usually destroyed by three weeks after transplantation. The graft site is composed primarily of lymphocytes, phagocytes and cellular debris, although some pituitary cells remain. This correlates well with the rapid rate of newt skin rejection by an axolotl host (Taban and Connelly, unpublished; see too Cohen, 1969; DeBoth, 1970).

Pituitaries of two-year old sexually active axolotls have large numbers of cells containing little granulation and numerous 150 mμ diameter vesicles (clear cells). In addition, the type II cells appear to be less numerous in these pituitaries. Finally, in the regions bordering blood vessels there is evidence of fusion between cells of different types which can be seen at both the light and electron microscopic levels.

After treatment with thyroxine, two-year old axolotls undergo metamorphosis and become sexually inactive (Prahlad and DeLanney, 1965; Prahlad, personal communication).

Pituitaries of two-year old sexually active axolotls have large numbers of cells containing little granulation and numerous 150mμ vesicles (clear cells). In addition, the type II cells appear to be less numerous in these pituitaries. Finally, in the regions near blood vessels there is evidence of fusion between cells of different types which can be seen at both the light and electron microscopic levels.

After thyroxine treatment, axolotls metamorphose and become sexually inactive (Prahlad, personal communication). Type II cells appear more frequently than in pituitaries of non-thyroxine-treated animals. Rough-surfaced endoplasmic reticulum is present in many pars distalis cells and clear cells and cell fusion are very noticeable.

The lack of detection of granule extrusion as a mode of secretion as well as the presence of numerous multitubular bodies in the axolotl pituitary has prompted the proposed secretory mechanism diagrammed in Fig. 49. It is felt that secretion in the axolotl pituitary occurs in a manner not easily detected by standard microscopic techniques.

By correlating these results with those of other workers on amphibian pituitary cytology several conclusions about the function of these cells may be reached. 1) The number of different cell types present in the axolotl pituitary suggests that there is no anatomical deficiency in this gland. 2) The axolotl pituitary is capable

of reacting to a new environment (newt) and does so more rapidly than after auto- or homotransplantation. 3) Since cell type III retains its granule content even up to four months after xenoplastic transplantation it is felt that this cell is a possible site for the production of a prolactin-like hormone. 4) The morphology of the type II cells and their fluctuations in relation to sexual cycle suggest that these cells are gonadotrophic in nature. 5) The morphology of the type I cells suggests that they correspond to type I bssophils of light microscopy and may be the site of TSH production. Possible interrelations between the various granulated and clear cells (chromophobes) is discussed. In addition, a discussion of the possibility of pituitary cells secreting more than one hormone during their life cycle is discussed.

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A THESIS

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

Department of Zoology

1970

ACKNOWLEDGEMENTS

The author would like to express sincere appreciation to Dr. C. S. Thornton for his support and encouragement of the work presented herein. His vast scientific experience was invaluable in the design and performance of these experiments.

A special thank you is also extended to Dr. S. K. Aggarwal, without whose advice and moral support this work could not have been accomplished. I particularly appreciated his willingness to act as Father Confessor and his offers of consolation when times were especially hard.

Dr. E. M. Rivera and Dr. B. E. Walker are thanked as well, for serving as members of my Guidance Committee and for thier advice and assistance in the preparation of this thesis.

It is especially pleasant for me to express public thanks to those fellow conspirators who helped make my stay in the Regeneration Laboratory so rewarding. I am grateful to Dr. Roy A. Tassava for teaching me so much about regeneration and hormones and for providing the experimental basis for this research. Special thanks are also extended to Dr. Charles Taban, Dr. Nick Shuraleff and Dr. Charles Tweedle, who have each added so much to the whole experience through their unique and

individual viewpoints on science and life in general. Thanks should also be expressed to Mrs. B. Henderson, who helped and taught me more than she may ever know.

I am grateful to my parents Mr. and Mrs. Tom Connelly for their unfailing support and encouragement throughout the many years before and during the performance of this research. Without their advice and guidance I would never have come this far.

Finally, I would like to express my sincerest thanks to my wife Jo Anne who suffered with me through all the failures, whose love helped to overcome them and to make the successes so much more worthwhile. Thanks Beagly.

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or globule which would be difficult to detect since the contents of the granule differ only slightly from those of the extracellular matrix. Pathway 5 represents a theoretical method for the formation of multitubular structures in the axolotl pituitary. Granules and large globules (lysosomes) fuse, undergo a change in electron density, and produce material(s) which might assume a multitubular conformation upon fixation. G = Golgi complex; S = secretory granules; GL = globule; MB = multitubular body. Please note again the substructure of the globules in the type II cell in Fig. 20 and Fig. 46.

Introduction

Interest in correlating the ultrastructure and function of the cells of the amphibian pituitary has increased greatly in recent years (Doerr-Schott, 1962, 1965, 1966, 1967, 1968; Cardell, 1964a, b, 1968; Dent and Gupta, 1967; Iturizza, 1964; Srebro, 1965; Masur, 1969; Masur and Holtzman, 1969; Bunt, 1969). Attention has been devoted particularly to the cytological analysis of the amphibian pituitary during development and metamorphosis and also during changes in the adult physiological state (van Oordt, 1968). However, electron microscopic studies of the pituitary in such an interesting deviation in metamorphosis as neoteny are less numerous (Lynn, 1961; van Oordt, 1968). It is thought that neoteny may be due to one or several of the following: failure of the pituitary to secrete sufficient thyroid-stimulating hormone (TSH); failure of the hypothalamus to produce sufficient amounts of TSH releasing factor (TSHRF); failure of the thyroid to secrete sufficient amounts of thyroxine, or an insensitivity of target tissues to thyroxine (Lynn, 1961; Lynn and Wachowski, 1951; Dent, 1968). Cytological analysis of the pituitary glands of some neotenic forms under various experimental conditions might help to determine which one or which combination of these possibilities is the cause of metamorphic inhibition.

The Mexican axolotl, Ambystoma mexicanum, is a

permanently neotenic uordele which retains its larval morphology both in the laboratory and in the wild (Lynn, 1961; Blount, 1950; New, 1966). Although the Mexican axolotl does not normally undergo spontaneous metamorphosis it may be induced to metamorphose by stimulating the thyroid in a variety of ways (Uhlenhuth and Schwartzbach, 1927; Lynn and Wachowski, 1951; Prahlad and DeLanney, 1965).

Although failure of the axolotl to metamorphose naturally has been attributed to a deficiency in the pituitary (Lynn, 1961; Prahlad, 1968) the experiments of Blount (1950) and Bytinski-Salz (1935) could point rather towards a malfunctioning of the hypothalamus. Pituitary gland rudiments from Harrison's stage 31 embryos of Ambystoma tigrinum transplanted ectopically to axolotl (A. mexicanum) embryos stimulated spontaneous metamorphosis in resultant larvae. Reciprocal heteroplastic pituitary grafts did not support normal spontaneous metamorphosis in A. tigrinum (Blount, 1950). However, these grafts also contained hypothalamic rudiments. These results have been interpreted to mean that the axolotl pituitary is deficient in some way which does not allow it to stimulate metamorphosis in A.tigrinum, a species which normally undergoes spontaneous metamorphosis (Blount, 1950; Lynn, 1961). The experiments of Bytinski-Slaz (1935), however, emphasize the importance of the hypothalamus for normal metamorphosis under conditions similar to those in the experiments of Blount (1950). Bytinski-Salz (1935) transplanted, orthotopically,

pituitary rudiments without hypothalamic rudiments from A. tigrinum embryos at stage 28 to A. mexicanum embryos. This did not result in spontaneous metamorphosis of resultant larvae, while reciprocal orthotopic grafts of A. mexicanum pituitaries to A. tigrinum did produce spontaneous metamorphosis of some host animals. These results cannot be adequately explained by degeneration of the graft and regeneration of the host's own pituitary as Blount (1950) had proposed. If this were the case, one would expect some similar results in the experiments of Blount (1950). These data seem rather to point to a malfunctioning of the axolotl hypothalamus with a resultant failure to produce TSHRF. As a consequence, insufficient TSH and little thyroxine would be produced and there would thus be no metamorphosis (Etkin, 1964, 1968). Thyroid activity in the axolotl appears to be sufficient to cause normal maturation of the hypothalamo-hypophyseal grafts from A. tigrinum larvae, while that of the tigrinum host does not appear sufficient to stimulate the maturation of pituitary-hypothalamic rudiments from A. mexicanum. Etkin (1968) has shown that the pituitary-hypothalamic axis is essential for completion of urodele metamorphosis. The hypothalamic malfunction may be remedied genetically (Humphrey, 1944, 1967) or may be corrected by treatment at almost any time during the axolotl's life cycle with a single dose of thyroxine (Prahlad and DeLanney, 1965, 1967; Prahlad, 1968).

Tassava (1969 a, b) has recently shown that an axolotl pituitary transplanted to the lower jaw of an hypophysectomized adult newt secretes hormones capable of maintaining the survival of the host. Similar results were obtained with ectopic pituitary autografts, or by treatment of the hypophysectomized newts with prolactin and thyroxine (Tassava, 1969a; Connelly et al., 1968a). The fact that the pituitary of the axolotl appears to have sufficient TSH activity to promote survival in the adult newt lends support to the belief that the metamorphic block in the axolotl is at some level other than that of the pituitary itself.

The lack of sufficient information concerning the cytological condition of the pituitary grafts in the experiments of Blount (1950), Bytinski-Salz (1935), and Tassava (1969b), or of pituitaries of axolotls treated with thyroxine (Prahlad and DeLanney, 1965, 1967; Prahlad, 1968) points up the need for a detailed cytological study of the axolotl pituitary gland. Furthermore, a transplantation experiment might prove to be a relatively simple method of identifying the functional activity of certain cell types within the pituitary. The transplanted pituitary is thought to have a high prolactin activity (Masur, 1969) and possibly a high TSH activity (Tassava, 1969a,b; Compher and Dent, 1970). Thus, it is possible that examination of the transplanted axolotl pituitary, particularly after xenoplastic grafting, might provide some information regarding the morphology of TSH and

prolactin secreting cells. Since an ectopically grafted axolotl pituitary promotes survival in hypophysectomized adult newts there is readily visible evidence of graft activity. It is felt that this relatively simple operation might provide interesting information regarding several different cell types.

The treatment of an axolotl with thyroxine induces a drastic change in the animal's morphology and physiology (Prahlad and DeLanney, 1965, 1967; Prahlad, 1968). Perhaps examination of the pituitaries of such thyroxine-treated axolotls would yield information regarding the TSH cells, particularly since thyroid activity increases markedly during metamorphosis (Prahlad, 1968).

Therefore, studies of the normal, transplanted and thyroxine-treated axolotl pituitary have been undertaken in order to attempt to determine the substructure of the axolotl pituitary and the effects of experimental treatment on its cellular morphology and activity.

Materials and Methods

Young (8 months old) laboratory raised Ambystoma mexicanum larvae (8-10 cm. body length) were used for description of the cells of the normal and transplanted pituitary. Animals were kindly provided by Dr. R.R. Humphrey, Department of Zoology, Indiana University. Larvae were fed brine shrimp and beef liver ad libitum and were

maintained in small individual aquaria containing aerated tap water at 21 ± 1° C. Operations were performed while animals were under MS 222 anasthesia (Tricaine methane sulfonate, Sandoz; 1:1000). Adult newts, Diemictylus viridescens, obtained from Lewis Babbitt, Petersham, Massachusetts, served as xenoplastic hosts or pituitary donors. These animals were kept in one gallon squat glass fish bowls, eight animals per bowl, in aerated tap water 21 ± 1° C. and fed beef liver ad libitum until one week prior to experimentation. Newts were then fasted until at least three weeks after hypophysectomy and implantation of an axolotl pituitary. This treatment was used to cause a maximum enhancement of the effects of hypophysectomy on newt survival (Tassava, 1969a, b). Axolotls used in autoplastic, homoplastic or reciprocal xenoplastic grafting experiments were subjected to this same feeding regimen even though hypophysectomy does not noticeably affect axolotl survival.

Anesthetized animals were placed on their backs under a dissecting microscope and their lower jaws held back with a pair of large, blunt forceps. An incision in the palatine mucosa was made with extremely sharp watchmaker's forceps, and the sella turcica thus exposed. A flap of cartilage (axolotls) or bone (newts) ventral to the pituitary was dislodged and the pituitary was removed with fine pointed watchmaker's forceps. The flap of bone

or cartilage was then replaced, although the presence or absence of this flap has no effect on animal survival (personal observation). The glands thus removed were then fixed or transplanted according to the experimental design. Hosts receiving a graft were thus deprived of a pituitary with normal connections to the hypothalamus (Fig. 7).

When pituitaries removed as described above were to be transplanted, they were placed in a small pocket beneath the skin of the lower jaw of the host. This pocket was formed by making an incision in the skin, sliding watchmaker's forceps into it between the skin and the underlying musculature, and then allowing the forceps to expand in the wound. This freed a small area of skin from the muscles beneath it and provided an easily visible site for transplanting glands. Pituitaries were transplanted autoplastically, homoplastically and xenoplastically with reciprocal xenoplastic grafts being performed also. Transplanted glands were removed at one week, three weeks or four months after transplantation. In cases where animals were not killed at the time of transplant removal, a record of survival of the resultant completely hypophysectomized hosts was kept.

Autografted and homografted glands were fixed at one week for electron microscopy, and at three weeks for light and electron microscopy. Xenoplastically transplanted axolotl pituitaries were fixed at one week for electron microscopy, three weeks for light and electron microscopy,

and four months for light and electron microscopy. Reciprocally grafted newt pituitaries were fixed three weeks after transplantation and were prepared only for electron microscopy. Normal glands were used as controls in all experiments, and were prepared for light and electron microscopy in exactly the same manner as the transplants. In all groups, three to six pituitaries were prepared for microscopic observation with either the light or the electron microscope. The majority of the normal pituitaries used for this study were fixed during the spring and early summer months. Some glands were fixed for light microscopy during the early winter months in an effort to estimate seasonal variation in the cells in the gland. Pituitaries in all transplant experiments were fixed in the spring and early summer months. Reciprocally transplanted newt pituitaries were fixed in the fall, as were those from two-year old axolotls and thyroxine-treated axolotls.

Pituitary glands to be used for light microscopy were fixed for 24 hours in sublimated Bouin-Hollande fluid, dehydrated through graded alcohols, cleared in xylene and embedded in paraffin. Some glands were dehydrated and embedded in glycol methacrylate according to the method of Ashley and Feder (unpublished). This provided easily obtainable thick sections (1u) with excellent cell detail,

cut on an ordinary rotary microtome. The plastic, although hygroscopic, presented some staining difficulties and did not allow for the same flexibility in staining as did sections of paraffin embedded material. Sections (4-5μ) of paraffin embedded pituitaries were stained differentially with PAS-alcian blue-orange G (Herlant, 1960) or with van Oordt's modification of the Cleveland-Wolfe trichrome (Cleveland and Wolfe, 1932; van Oordt, personal communication). Before staining, sections were treated with Lugol's solution to remove mercuric deposits. Only four to five sections were placed on each slide to allow differential staining of closely related sections with several techniques.

A variety of fixatives for electron microscopy were employed in order to determine the optimum method of fixation for the axolotl pituitary. Pituitary glands from young animals were fixed in glutaraldehyde (Sabatini et al., 1963) in percentages ranging from 1%-3% in cacodylate buffer ranging in molarity from 0.025 M - 0.1 M. The osmotic strength of the various fixatives varied from 200 mosmol to 450 mosmol. Optimum osmotic strength was determined to be approximately 390-400 mosmol. Pituitaries were also fixed in an iced mixture of glutaraldehyde (1%) and osmium tetroxide (1%) in a 0.1M cacodylate buffer according to the method of Franke et al. (1969). Optimum reproducibility of fixation was obtained, however, when

glands were fixed for 1½ hours at 0-4°C. in 1% osmium tetroxide and 1% potassium dichromate in 0.1M sodium cacodylate buffered at pH 7.4 . Dehydration was carried out rapidly through a graded series of acetone to propylene oxide (Luft, 1961) followed by embedding in Araldite (Durcupan) or Araldite 502 (Sema Co., Baltimore). Ultrathin sections cut using an LKB Ultratome III were picked up on 75 mesh formvar coated copper grids, stained with uranyl acetate and lead hydroxide, and viewed under an Hitachi HU 11-E electron microscope at an accelerating voltage of 50 or 75 kV.

Glands from reproductively active two-year old axolotls were removed from animals kindly provided by Mr. E.D. Pollack, Department of Zoology, University of Iowa. These pituitaries were prepared in a fashion similar to that described above. Three animals were used for light microscopy and three for electron microscopy. Pituitary glands from thyroxine-treated axolotls were fixed and embedded for electron microscopy according to the above scheme by Dr. K.V. Prahlad, Department of Biological Sciences, Northern Illinois University and sent to me. The fixation of pituitaries from thyroxine-treated animals was performed three to four months after the completion of metamorphosis induced according to the methods of Prahlad and DeLanney (1965). Animals thus treated were moulting at the time of fixation and were known to have active thyroids (Prahlad, 1968).

For purposes of orientation and correlation, thick sections (1μ) adjacent to these thin sections were stained with 1% methylene blue-1% borax in aqueous solution. Unfortunately such thick sections of epoxy embedded pituitaries could not be reliably stained differentailly even after removal of the plastic. Several different methods were attempted, but the results were so variable that differential staining of epoxy sections was not routinely attempted. This is a problem which has plagued pituitary cytologists for some time and which may eventually by solved satisfactorily (van Oordt, personal communication; Heath, personal communication).

Sections of glycol methacrylate embedded pituitaries were stainable reliably only with PAS and methylene blue. Acid dyes such as orange G and erythrosine would not penetrate the plastic consistently. Many different techniques were tried for forcing these dyes through the plastic, but none were successful. Although some authors (Bunt, 1969; Doerr-Schott, 1966) have reported successful differential staining of thick sections from which the epoxy resin has been removed, it has been this author's experience that staining of such sections is extremely difficult and highly variable. There is no apparent way to remove glycol methacrylate from tissues once sections have been cut, although similar treatments will dissolve epoxy resins. Therefore, direct correspondence between

cells seen in differentially stained sections of paraffin embedded material and those seen under the electron microscope is not possible at this time.

Results

Normal Young Axolotl Pituitaries

Differentially stained sections of paraffin embedded material reveal that the pituitary of the axolotl can be divided into three easily recognizable regions, the pars nervosa (PN), the pars intermedia (PI), and the pars distalis (PD), (Fig. 1, 2, 3,). The pars nervosa stains intensely with PAS or with alcian blue (pH 0.2). The cells of the pars intermedia stain weakly with PAS or with aniline blue. The uniform staining reaction of the cells of the pars intermedia as well as a scarcity of blood vessels in this region make the separation between it and the pars distalis very clear. The cells of the pars distalis show a highly varied staining. Some cells stain with PAS, others stain with alcian blue (pH 0.2) and may contain large, PAS-positive globular inclusions (Fig. 4). Finally, there are some cells that stain with orange G (Fig. 5). If the sections are stained with Cleveland-Wolfe's (1932) trichrome, there appear some purely aniline blue-positive cells (sky blue), some cells which stain with both aniline blue and an acid dye (orange G or erythrosine; purple cells) (Fig. 6), and others which stain with orange G or erythrosine. No differentiation between orangeophils and erythrosinophils

Figure 1. Low power light micrograph of a median sagittal section of an axolotl pituitary fixed in the spring-early summer months. PN, pars nervosa; PI, pars intermedia; PD, pars distalis. All cell types are randomly distributed through the pars distalis. See also Fig. 3. PAS-alcian blue-orange G (Herlant, 1960).

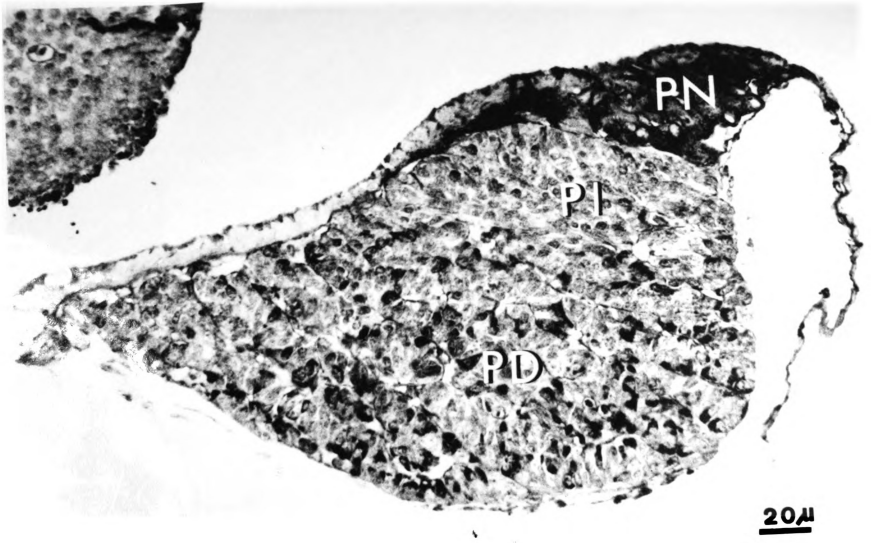
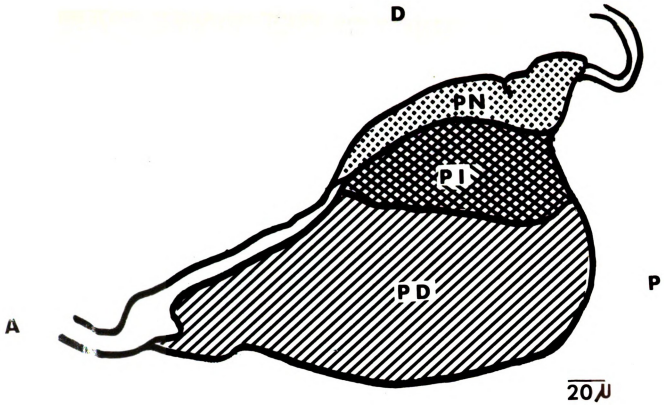


Figure 1

Figure 2. Line drawing of a medial sagittal section of a pituitary showing more clearly the division of the pituitary into three regions. The staining reactions of the cells in each region are shown below the drawing.

PD, pars diatalis
PI, pars intermedia
PN, pars nervosa
A, anterior
P, posterior
D. dorsal

AB, Alcian blue
AnB, Aniline blue
PAS, periodic acid Schiff's
OG, Orange G
ERY, erythrosine



PN = AB^{+}, PAS^{+}

PI = $PAS^{\pm}$, $AnB^{\pm}$

PD = PAS^{+}; $AB\ \&\ PAS^{+}$; $AnB\ \&\ OG^{+}$; OG^{+}
ERY? AnB^{+}

Figure 2

Figure 3. Low power light micrograph of a median sagittal section of an axolotl pituitary similar to that in Fig. 1. PAS-alcian blue-orange G (Herlant, 1960).

Figure 4. Alcian blue-positive cell containing large PAS-positive globules in the pars distalis of a young untreated animal (PAS-alcian blue-orange G, Herlant, 1960; 1500X).

Figure 5. Aniline blue-positive and orange G positive cells in the pars distalis of a young untreated axolotl. (Cleveland-Wolfe, 1932 trichrome, 1800X).

Figure 6. A large group of purple cells in the pars distalis of a young untreated axolotl (Cleveland-Wolfe, 1932 trichrome, 1800X).

Figure 7. Low power light micrograph of an axolotl pituitary in the lower jaw of an hypophysectomized adult newt. Note the arrangement of cell cords in the gland, the integrity of the transplant and its position between the muscle (above) and the skin (below) of the host. (400X)

Figure 8. Low power light micrograph of a differentially stained three-week axolotl pituitary xenograft. Note the extensive degranulation of pituitary cells (compare with Fig. 11, 12) and almost complete absence of basophilic cells. Arrows indicate the thick connective tissue capsule surrounding the transplant (Cleveland-Wolfe, 1932 trichrome; 400X).

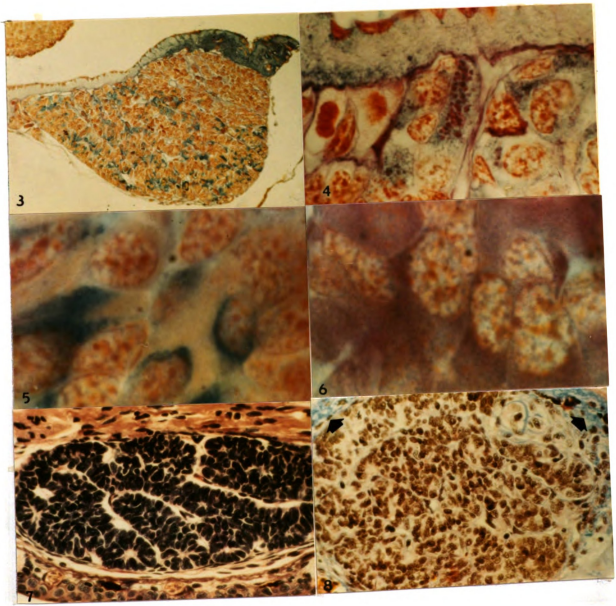

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Figure 9. Low power light micrograph of a 4-month old xenoplastically grafted axolotl pituitary. Arrow indicates metachromatic cytoplasm. Methylene blue, glycol methacrylate embedded. (400X).

Figure 10. Light micrograph showing orangeophilic cells and cells with large PAS-positive globules in a 4-month old xenoplastically grafted axolotl pituitary (alcian blue-PAS-orange G; Herlant, 1960; 1800X).

Figure 11. Low power light micrograph of a 3-week old axolotl pituitary autograft stained with Cleveland-Wolfe's (1932) trichrome. Note the healthy appearance of the gland and the absence of a thick connective tissue sheath (see Fig. 8 for comparison to xenograft; 400X).

Figure 12. Low power light micrograph of a 3-week axolotl pituitary homograft stained with Cleveland-Wolfe's (1932) trichrome. Note the preponderance of acidophils in this graft (orange cells) and the absence of a heavy connective tissue sheath surrounding it (see Fig. 8 for comparison to xenograft; 400X).

Figure 13. Light micrograph of orange G-positive cells and purple cells (basophils) in a 3-week homografted axolotl pituitary. These basophilic cells are almost completely absent in 3-week xenografts (Cleveland-Wolfe, 1932 trichrome; 1800X).

Figure 14. Light micrograph of a zone of cell fusion in the pars distalis of a thyroxine-treated axolotl. Note the lack of distinct cell boundaries (1800X).

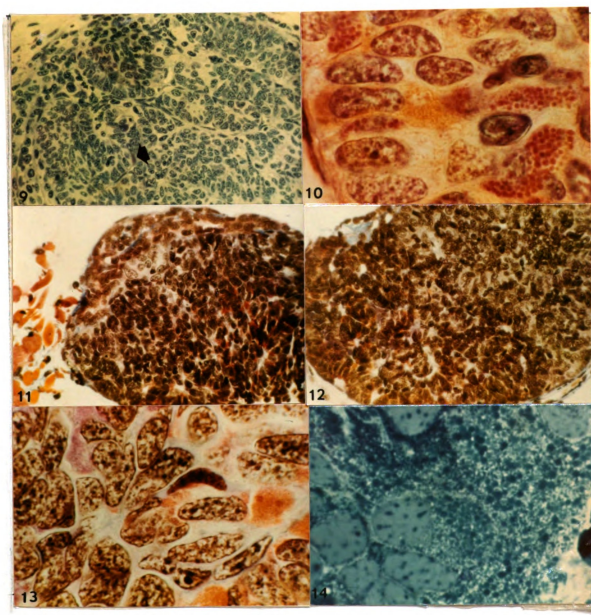
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Figure 15. Line drawing of a median sagittal section of a pituitary fixed during the early winter months, showing the regionalization of cells within the pars distalis.

PAS, periodic acid Schiff's

ERY, erythrosine

AB, alcian blue

A, anterior

P, posterior

D, dorsal

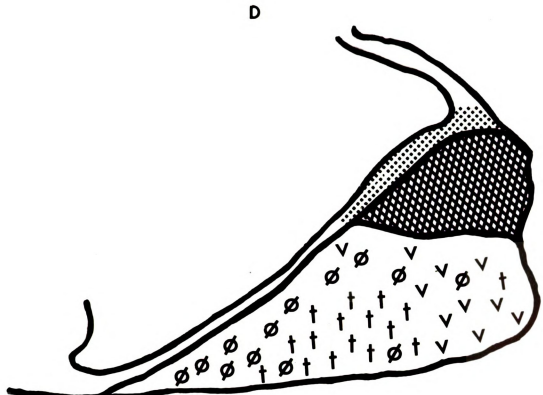


Figure 15

has been obtained. In glands which are fixed in the spring or early summer months, no obvious regionalization of the cell types can be seen in the pars distalis. In pituitaries fixed during the early winter months, a more obvious arrangement of the cells within the pars distalis can be found. PAS-positive cells tend to be concentrated in the rostral region, erythrosinophils (orangeophils) in the ventro-medial region, and the alcian blue-positive cells in the caudal region beneath the pars intermedia (Fig. 15). An arrangement of cells similar to this has been reported for the pituitary of A. tigrinum by Roofe (1937).

Under the electron microscope, the pars nervosa (Fig. 16) is easily identifiable since it is composed of large numbers of nerve fibers which contain neurosecretory granules of uniform size and relatively uniform electron density.

The cells of the pars intermedia are characterized by the absence of granular contents in their cytoplasm and by the presence of numerous vesicles (200-220 mμ in diameter) filled with a flocculent material (Fig. 17, 21c). The cells are ovoid to cuboidal in shape and their nuclei are generally centrally located. In many cells a well developed Golgi complex is present. Frequently large spaces appear between the cells of the pars intermedia. This appearance is not uncommon, as it has also been reported in the pars intermedia of the rat (Howe and Maxwell, 1968). In fact,

Figure 16. Electron micrograph showing the interface between the pars nervosa (PN), pars intermedia (PI) and the pars distalis (PD) of a young untreated axolotl. Note the nerve fibers and neurosecretory granules in the pars nervosa.

C, capillary

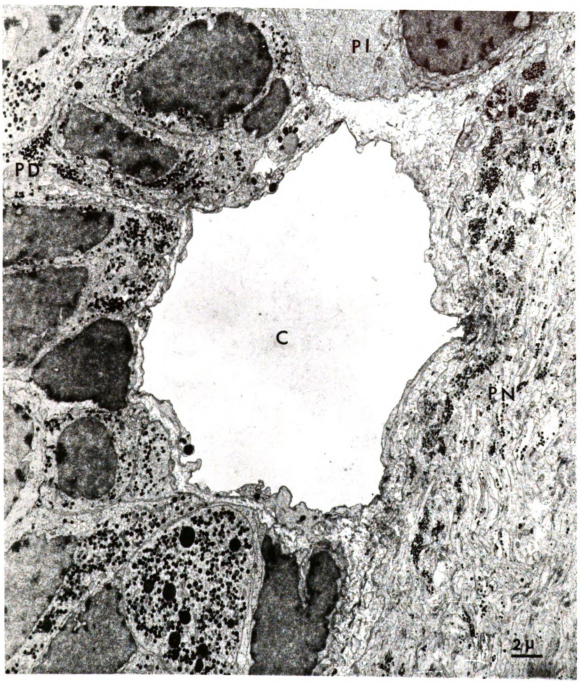


Figure 16

Figure 17. Electron micrograph of the interface between the pars intermedia (PI) and the pars distalis (PD) of a young untreated axolotl. Note the absence of granulation and predominance of vesiculation in the cells of the pars intermedia. Arrow indicates a space between cells of the pars intermedia. Two type IV cells are visible in the pars distalis.

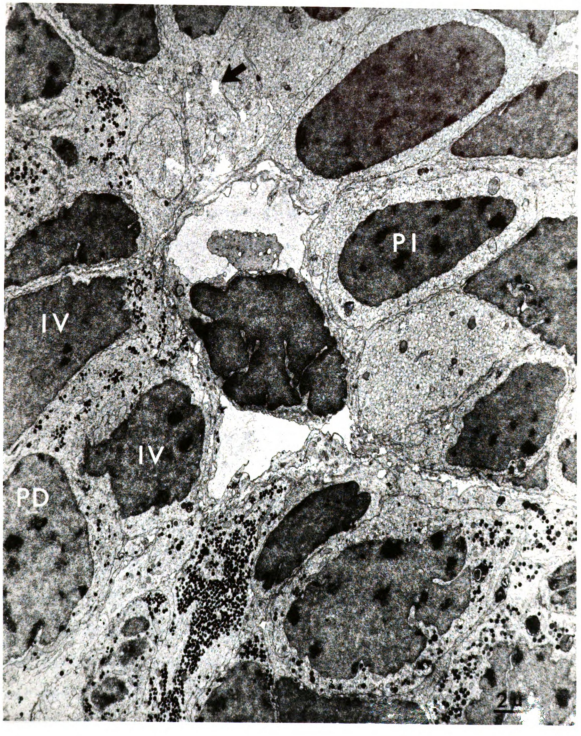


Figure 17

the cells of this region of the axolotl pituitary resemble some cells of the pars intermedia of the rat (Ziegler, 1963; Kurosumi et al., 1961).

At the electron microscopic level, the cells of the pars distalis may be divided into five different cell types based on their cytoplasmic contents (Fig. 18, 19, 20).

Type I cells contain secretory granules 260-330 mμ in diameter which vary in their electron opacity.

Type II cells contain secretory granules 330-520 mμ in diameter which are of variable electron opacity. In addition, large, dense globules and oval vesicles measuring 1μ and 130 x 330 mμ in diameter respectively are also present. Both cell types I and II oval to cuboidal in shape, with eccentrically placed nuclei.

Type III cells are particularly conspicuous due to their uniformly sized (330 mμ in diameter) and dense granular contents. These cells can be oval to spindle shaped.

Type IV cells are the least numerous of any of the cell types and are recognized by the lack of granulation in their cytoplasm and the presence of numerous vesicles approximately 220 mμ in diameter.

Type V cells correspond to the stellate cells of the newt pituitary described by Cardell (1964a, 1968, 1969). They are recognized by their triangular shape, their long cytoplasmic processes which are sent out between other pituitary cells, and by the numerous microfibrils present

Figure 18. Electron micrograph of the pars distalis of a young untreated axolotl showing the presence of cell type I, II, III and V. In addition, the arrow indicates large globule in type II cell.
N, nucleus of endothelial cell

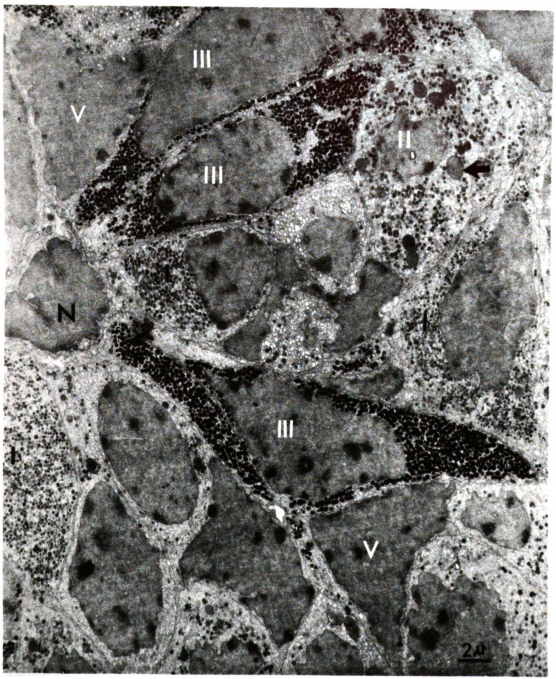


Figure 18

Figure 19. Electron micrograph of cell types I, II, III and IV in the pars distalis of a young untreated axolotl. Arrow indicates globule within a type II cell.

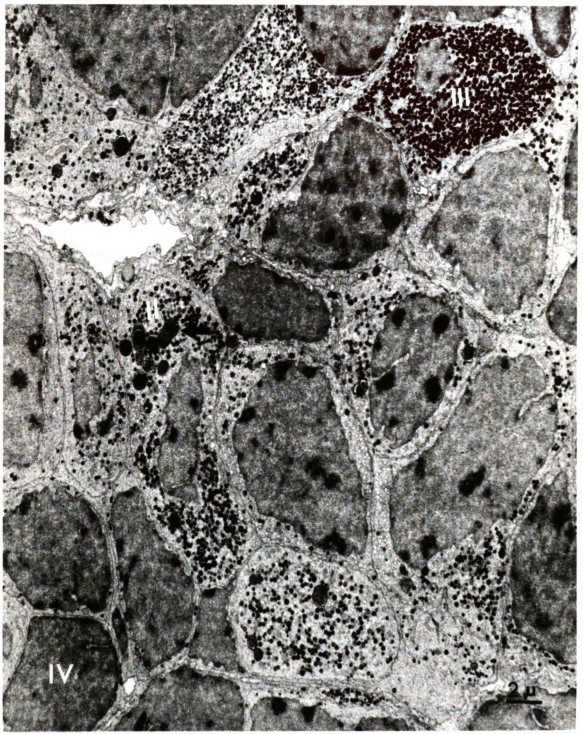


Figure 19

in these processes (Fig. 20).

Small clusters of ribosomes are occasionally found within the cytoplasm of the cells of the pars distalis, but no extensive network of rough-surfaced endoplasmic reticulum has been seen within these cells in such young animals. A well developed Golgi complex is also frequently seen in all of the cell types. The cells of the pituitary are in intimate contact and numerous desmosomes may be seen in both the pars distalis and the pars intermedia (Fig. 20). Numerous elongate mitochondria are present among the granular elements of the pars distalis. The secretory granules are membrane bound (Fig. 21b) and granules in the process of formation may often be found in the region of the Golgi complex (Fig. 20 inset).

In addition to the vesicles, granules and globules present within the cells of the axolotl pituitary, numerous large, membrane-enclosed multitubular bodies may often be found (Fig. 21a, b, c). These bodies may take several shapes, including irregular membrane clusters, and aggregates of a distinct tubular nature. Tangential sections through such tubular aggregates reveal a definite periodicity to the tubules themselves, suggesting that they could be stacks of uniformly shaped membrane rings (Fig. 21c). These structures are probably very similar to the tubular structures described in the pituitary of Triturus marmoratus by Doerr-Schott (1966) and the X-bodies described by Bunt (1969) in the pituitary of Taricha torosa.

Figure 20. Electron micrograph demonstrating the relationship between a type V cell, two type II cells and a type III cell. Arrows indicate desmosomes between the type V cell and other cells. Note the length of the processes of the type V cell and the presence of microfibrils (MF) within these processes. Note the substructure visible in the globules (GL) of a type II cell. Inset shows granules being formed in the region of the Golgi complex.

M, elongated mitochondrion

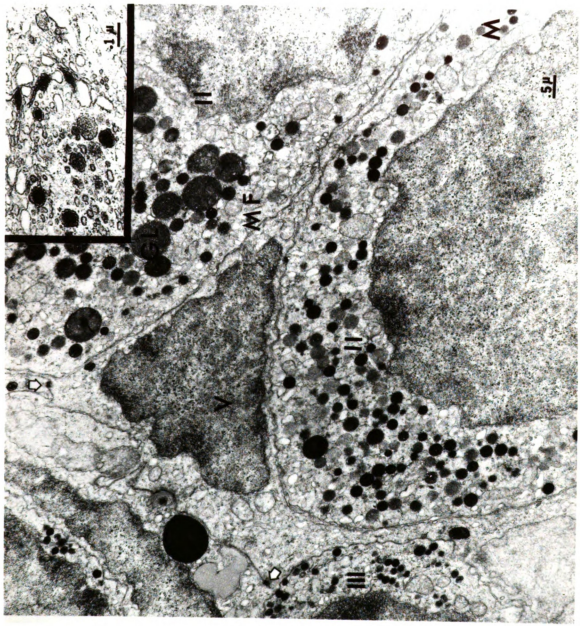


Figure 20

Figure 21a, b, c. Electron micrographs demonstrating the variety of forms which the multitubular bodies (MB) may assume. (a) and (b) are micrographs of multitubular bodies in a type I or type II cell in the pars distalis. The arrow in (b) indicates the membrane enclosing a secretory granule. (c) is a micrograph of multitubular bodies in a cell of the pars intermedia. The dark arrows indicate tangential sections of tubules in which periodicity may be seen. Cross sections of tubular elements may also be seen within these bodies. White arrow indicates a vesicle in the pars intermedia cell and the flocculent material present in it.

Figure 21a

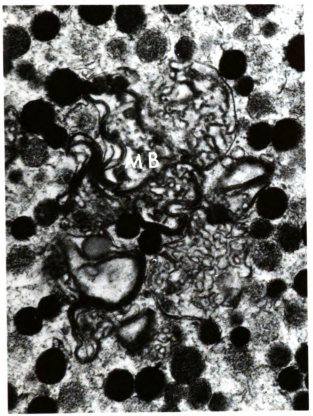
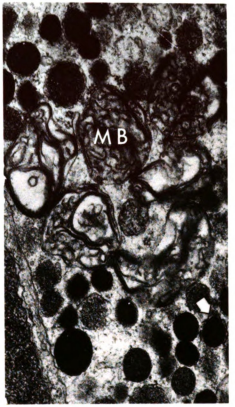


Figure 21b



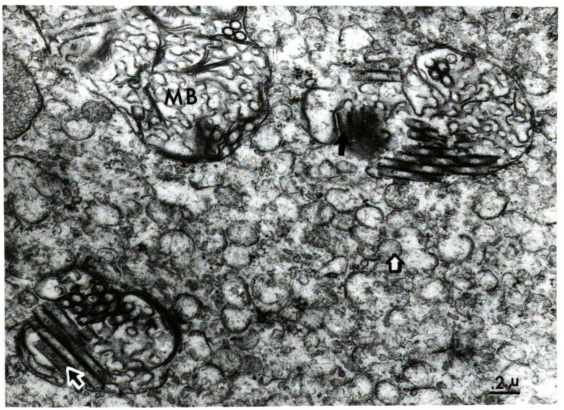


Figure 21c

Xenoplastic Transplants Axolotl to Newt

One Week Xenografts

The entire transplant at one week is encapsulated by a thin connective tissue covering (Fig. 7). Some phagocytic cells are present in the areas near the gland surface where cells injured by the transplantation procedure are being removed. The pars nervosa was never found in the transplant region, and thus must have degenerated shortly after transplantation or not have been transplanted at all. Of the two possibilities, the former is the most likely, since glands removed and fixed rather than transplanted always carry some portion of the pars nervosa.

The cells of the pars intermedia display a marked increase in activity as evidenced by the appearance of large amounts of rough-surfaced endoplasmic reticulum in their cytoplasm (Fig. 22). There is an increase in the number of mitochondria present in these cells as well as a slight increase in the frequency of appearance of electron dense granules. The Golgi complex appears as a well developed system of vesicles and cisternae.

The cells of the pars distalis remain in approximately the same condition as those of the normal gland (Fig. 23, 24). There is, however, an appearance of large clusters of granules within some cells which resemble phagocytic vacuoles (Fig 24). Cell type I has decreased slightly in

Figure 22. Electron micrograph of a pars intermedia cell in an axolotl pituitary one week after xenoplastic grafting. Note the presence of large amounts of rough-surfaced endoplasmic reticulum (RER) in these cells. Arrow indicates the Golgi complex of a pars intermedia cell. Some electron-dense granules may also be seen in the cytoplasm of these pars intermedia cells.
L, lipid inclusion.

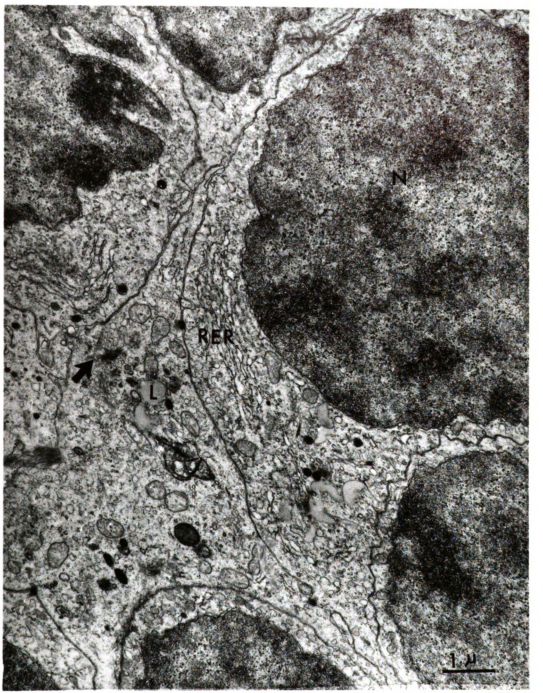


Figure 22

Figure 23. Electron micrograph of pars distalis cells of an axolotl pituitary one week after xenoplastic transplantation. Portions of a type II and a type IV cell may be seen. The arrow indicates a tangential section through a centriole or basal body.

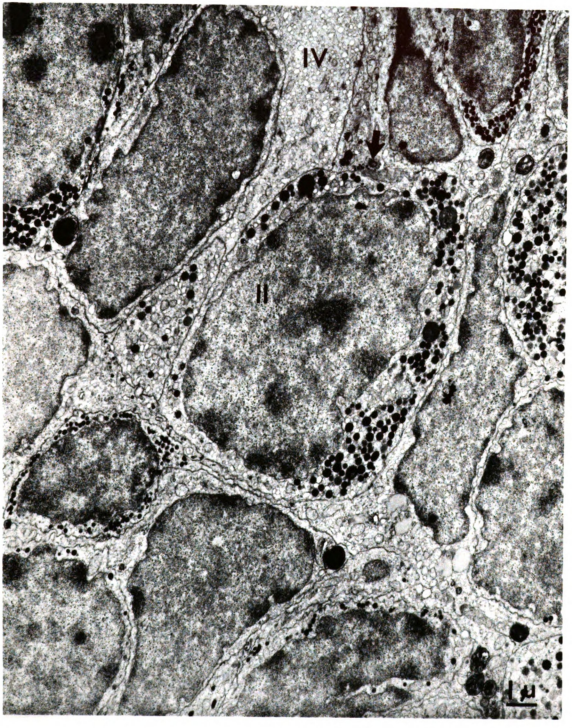


Figure 23

Figure 24. Electron micrograph of pars distalis cells in a one-week xenoplastic axolotl graft. Note the appearance of autophagic or phagocytic vacuoles (AV) in this region. A type III cell is particularly prominent.
CW, capillary wall
RBC, red blood corpuscle.

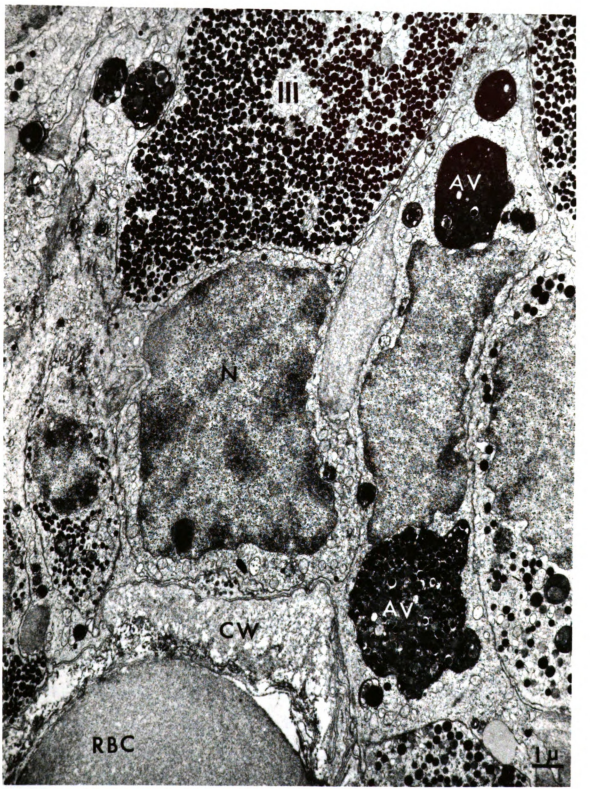


Figure 24

in frequency, but types II, III, IV, and V remain unchanged. Very small amounts of rough-surfaced endoplasmic reticulum may be seen, but this system is not developed to the same extent as that found in the cells of the pars intermedia.

The appearance of rough-surfaced endoplasmic reticulum in the pars intermedia is probably correlated with excessive skin darkening in the host animals. This is due to an increased secretion of melanocyte-stimulating hormone (MSH) by the pars intermedia which has been freed from hypothalamic inhibition (Jorgensen and Larsen, 1963; Jorgensen, 1968). The skin darkening occurs from five to seven days after transplantation and provides evidence that the gland has become established at its new site and is releasing its secretory products into the host's blood stream.

Three Week Xenografts

At three weeks after the operation, the transplanted gland is covered by a thick connective tissue sheath (Fig. 8). There are some phagocytic cells in the region of the graft beneath this sheath, but for the most part the transplant is composed of cells of an obvious pituitary nature. There is, by this time, a significant degranulation of cells in the pars distalis and classification of cell types relative to the normal becomes extremely difficult. Cell types I and III are

most often recognizable (Fig. 25, 26, 27, 28). Most cells are filled with large numbers of vesicles and many granules which appear to be in the process of breaking down. Some cells which have retained their granules have cisternae and rough-surfaced endoplasmic reticulum in their cytoplasm. The number of cells containing lytic bodies has increased, particularly in the region of the transplant surface. Identification of the cells in the graft as pituitary cells is relatively easy due to the organization of these cells in cords and their shape. There is no indication of granule release at the cell surface in any of the cells in the transplant either in the internal regions of the graft or in areas near vascular elements which nourish the graft.

Survival records of animals kept after the axolotl pituitary transplant is removed show that all the animals are dead by 21 days after complete hypophysectomy. This survival curve is closely parallel to that obtained when an adult newt is hypophysectomized and fasted without receiving any hormone replacement therapy (Tassava, 1969a, b; Connelly et al., 1968a; Fig. 29).

Four Month Xenografts

At four months, the transplant is composed of a surface covering of connective tissue, an intermediate layer of phagocytes, debris and cells of an epithelioid nature, and cells of an obvious pituitary nature (Fig. 9).

Figure 25. Electron micrograph of the pars distalis of a three-week axolotl pituitary xenograft. Note the difficulty in identifying the cell types present in this region.

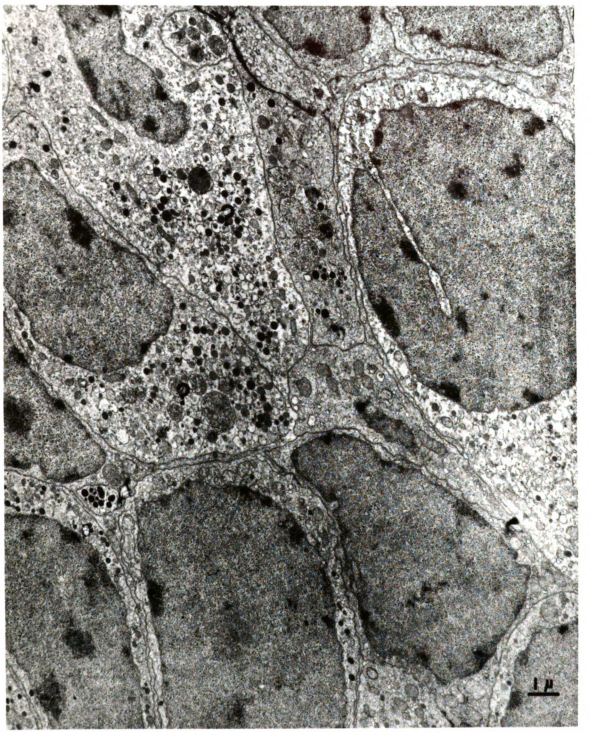


Figure 25

Figure 26. A type III cell present in the parsdistalis of a three-week axolotl pituitary xenograft.

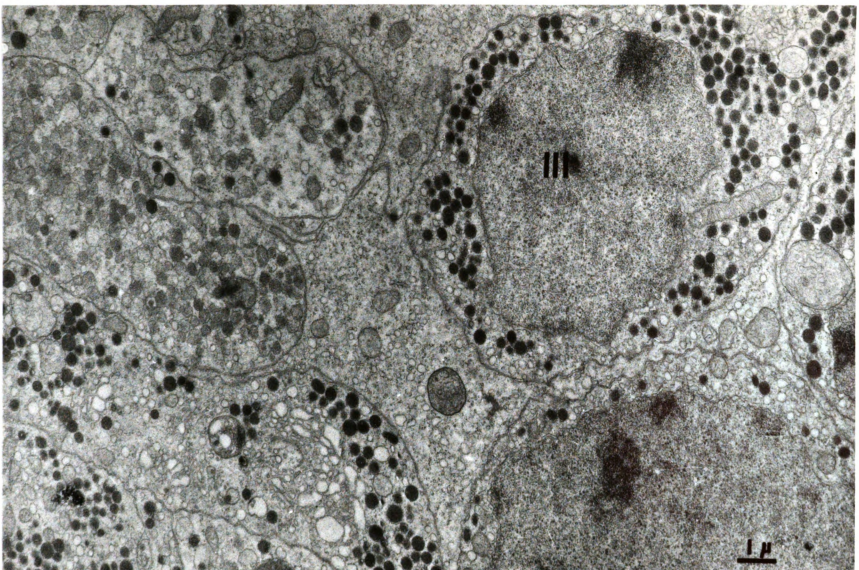


Figure 26

Figure 27. Type I cells in the pars distalis of a three-week axolotl pituitary xenograft.

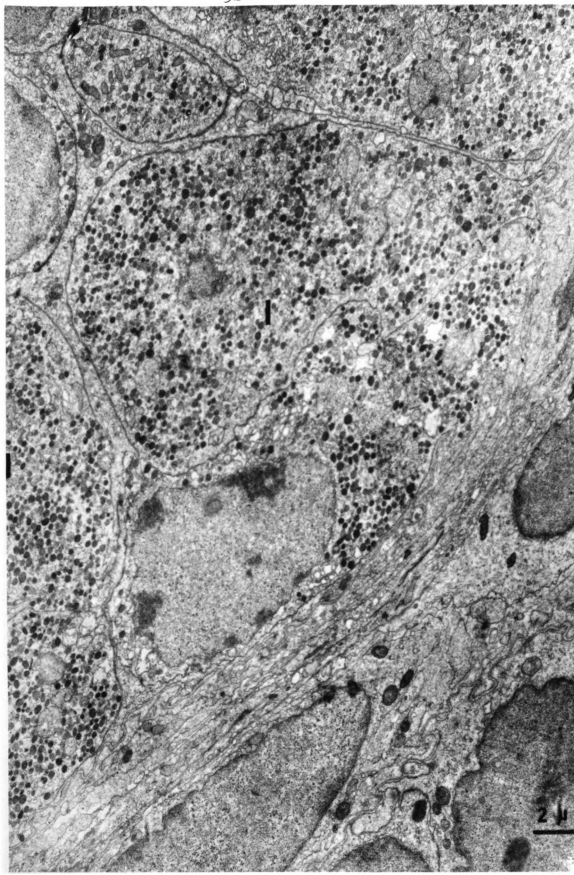


Figure 27

Figure 28. Electron micrograph of a type II cell and possibly a type III cell near a degranulating(?) cell containing multitubular bodies (MB) in the pars distalis of a three-week axolotl pituitary xenograft.

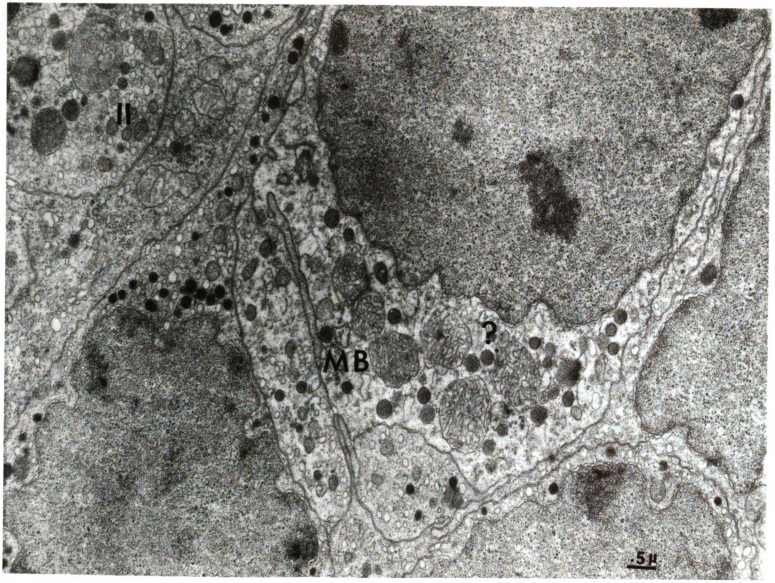


Figure 28

Figure 29. Survival records of fasted newts hypophysectomized at day 0 (▲---▲) and those of animals which have been fasted and provided with an ectopic axolotl pituitary until day0 (o——o) three weeks after original implantation of the graft. Note the similarity of the two curves. This survival rate indicates: (1) the functional replacement value of the axolotl graft; (2) the complete hypophysectomy of the animals receiving the graft. % SURV, percen surviving.

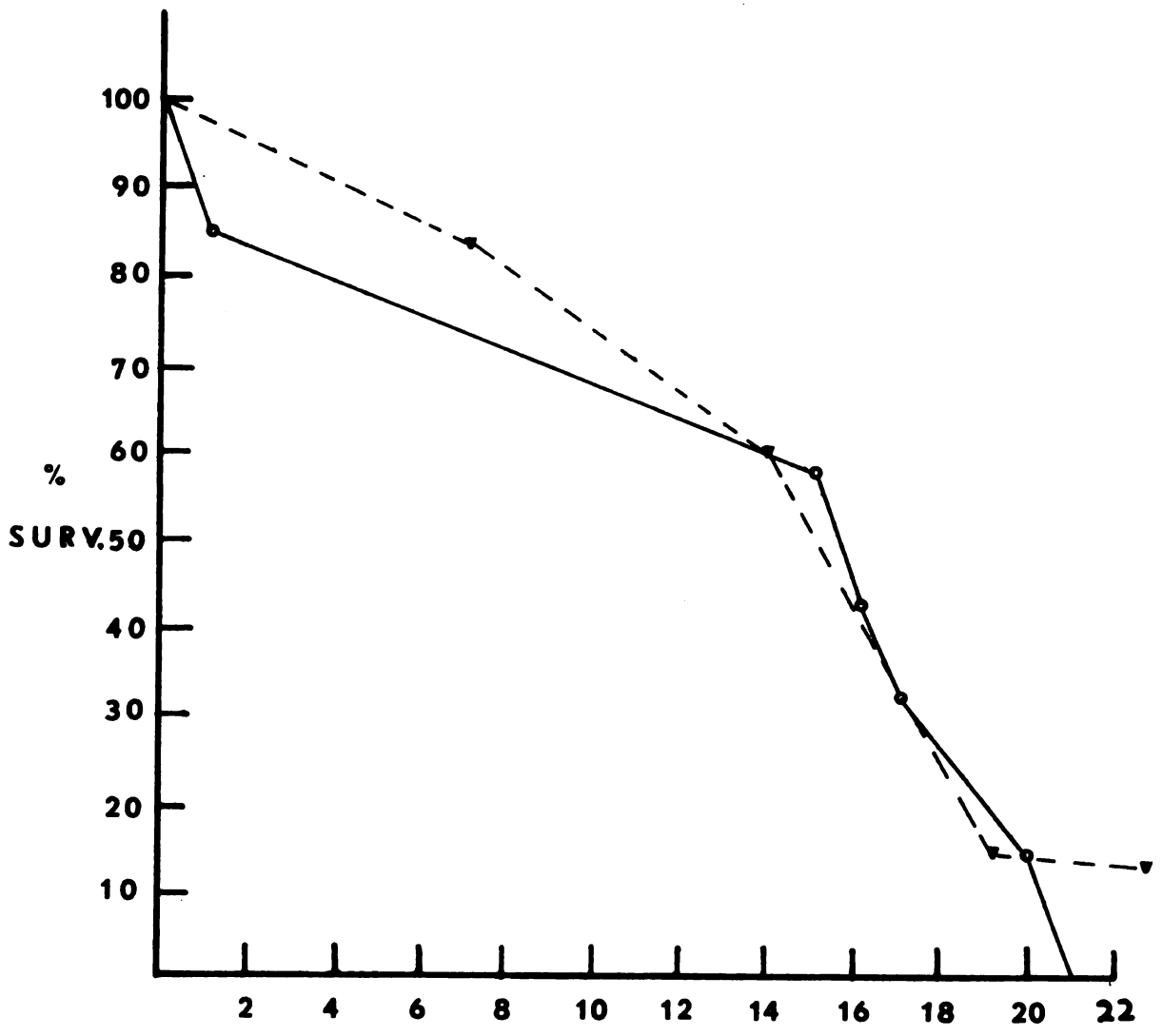

100
90
80
70
60
%
SURV.50
40
30
20
10
2
4
6
8
10
12
14
16
18
20
22
DAYS POST HYPOX

Figure 29

The pituitary cells themselves contain large globular inclusions or lytic bodies, or vesicles, granules and rough-surfaced endoplasmic reticulum. The development of the rough-surfaced endoplasmic reticulum in most of the glandular cells in these transplants is extensive and often takes the form of large cisternae. The granules present in those cells which still contain them are heavily electron dense. In this respect, they resemble those of the type III cells of the normal gland (Fig. 10,30, 31, 32). Granules in the process of formation may occasionally be seen in the region of the Golgi complex and occasionally cilia or centrioles may be seen. Mitotic figures are evident in these transplants, but occur mainly in the peripheral areas of recognizable pituitary cells (Fig. 33).

Auto- and Homografted Pituitaries

One Week Autografts

One week after ectopic transplantation, autografted pituitaries show the appearance of cells with lytic bodies in the peripheral regions of the graft beneath the connective tissue surrounding it. The cells of the pars intermedia appear activated as in the xenoplastic grafts at the same time period. The animals are excessively dark, as are xenoplastic hosts. The cells of the pars distalis have undergone some degranulation, particularly the type I and type II cells, but all cell types are easily recognizable (Fig. 34). There is no significant change

in the amount of rough-surfaced endoplasmic reticulum in the cells of the pars distalis.

Three Week Autografts

Three weeks after transplantation, autografted pituitaries show an increased degranulation of cells in the pars distalis. The cells of the pars intermedia appear to be more numerous. Those cells containing granulation and recognizably pars distalis cells do not appear to have been activated to increased synthetic activity by the transplantation. The degranulation which has occurred is less massive and widespread than that which occurs in the pars distalis after xenoplastic transplantation (Fig. 8, 11, 34). Although there is the appearance of some rough-surfaced endoplasmic reticulum in the pars distalis, it is not extensive.

One and Three Week Homografts

The homoplastic graft provides a particularly good control for determining the effects of an immunologic response of the host on graft cells. If changes in cellular structure and activity seen in xenografts were due to rejection of the graft by the host, comparable effects should result from an homograft response. The same conditions observed in autoplastically grafted glands prevail in homografts. The amount of degranulation which occurs is again less pronounced than that which occurs in xenoplastic grafts, particularly at three weeks

Figure 30. Electron micrograph showing a type III cell among degranulated cells in the pars distalis of a four-month old axolotl pituitary xenograft. The arrow indicates small clusters of rough-surfaced endoplasmic reticulum within the type III cell. The presence of large amounts of rough-surfaced endoplasmic reticulum (RER) in degranulated cells indicates that these cells are active and are not dying.

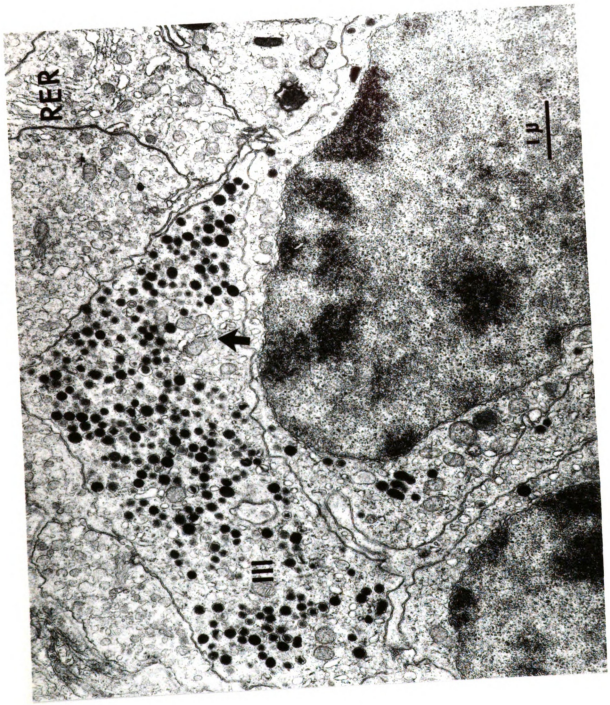


Figure 30

Figure 31. Degranulated cells in a four-month old axolotl pituitary xenograft. Note large globular structures, large amounts of rough-surfaced endoplasmic reticulum and granules forming in the Golgi complex (center).

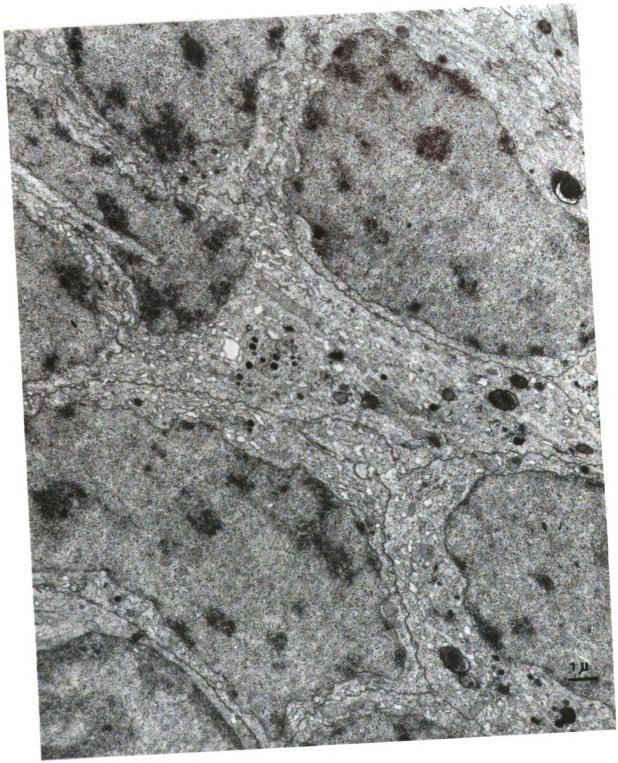


Figure 31

Figure 32. Electron micrograph of a four-month old axolotl pituitary xenograft showing cells with large lytic bodies and some with large cisternae of rough-surfaced endoplasmic reticulum.

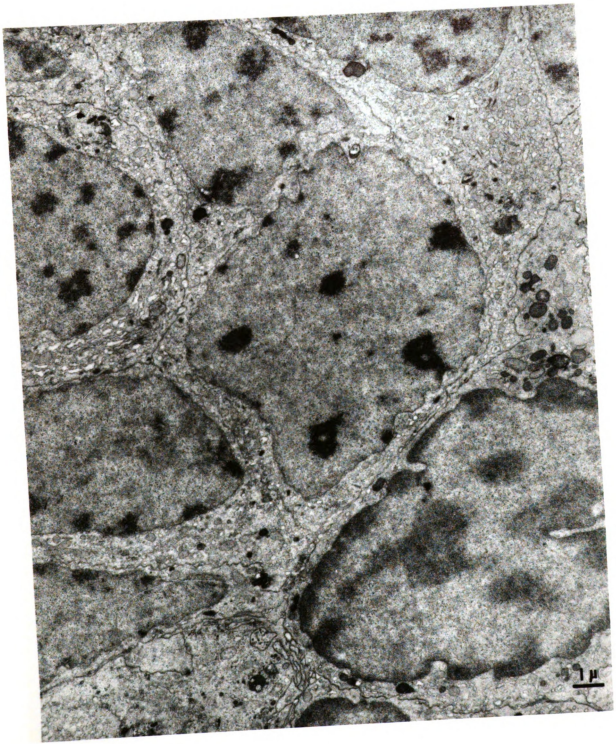


Figure 32

Figure 33. Electron micrograph of a mitotic figure near the surface of a four-month old axolotl pituitary xenograft. CH, chromosome.

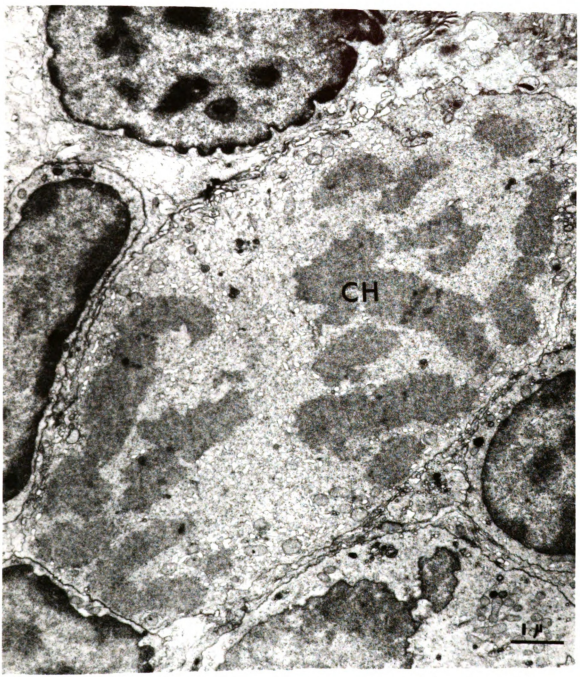


Figure 33

Figure 34. Electron micrograph of the pars distalis of a one-week autografted axolotl pituitary. Note slight degranulation of the type II cells. Several type III cells are also present.

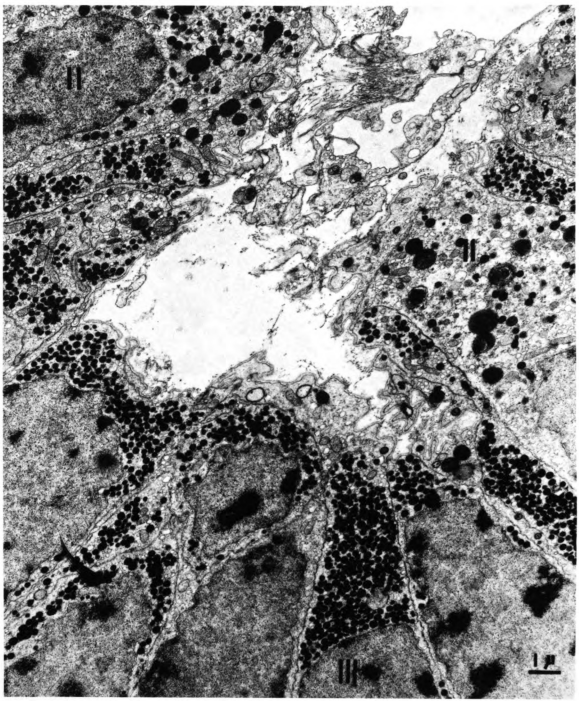


Figure 34

Figure 35. Electron micrograph of cells of the pars distalis of a three-week homograft showing nearly normal appearance of type II cells and a type III cell. Arrow indicates a section through a centriole.

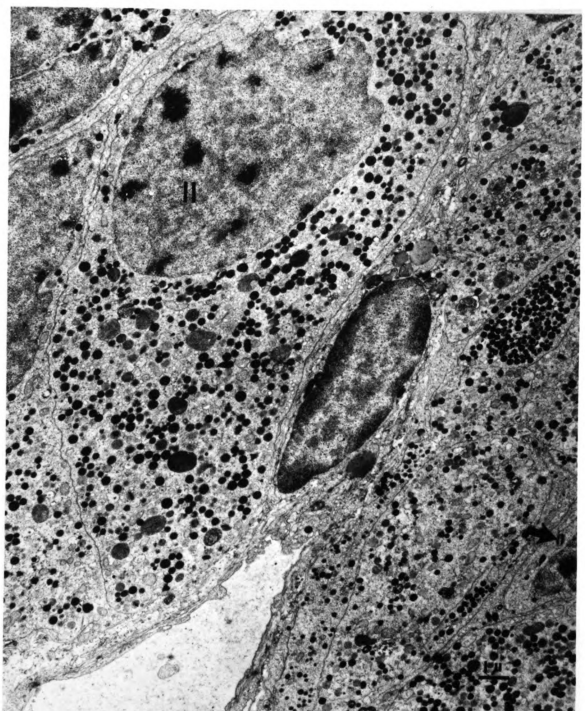


Figure 35

Figure 36. Electron micrograph illustrating the normal appearance of type III cells in a three-week axolotl pituitary homograft.

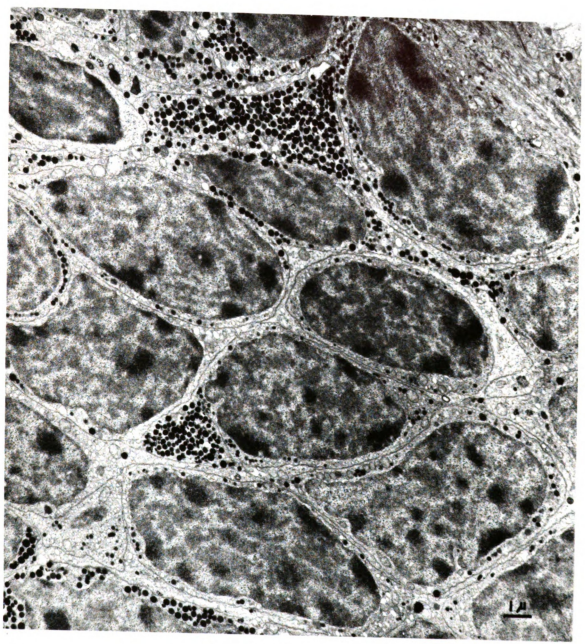


Figure 36

Figure 37. A clear cell of an intermediate between a granulated and a clear cell (CC) in a three-week axolotl pituitary homograft. Note the type III cells nearby.

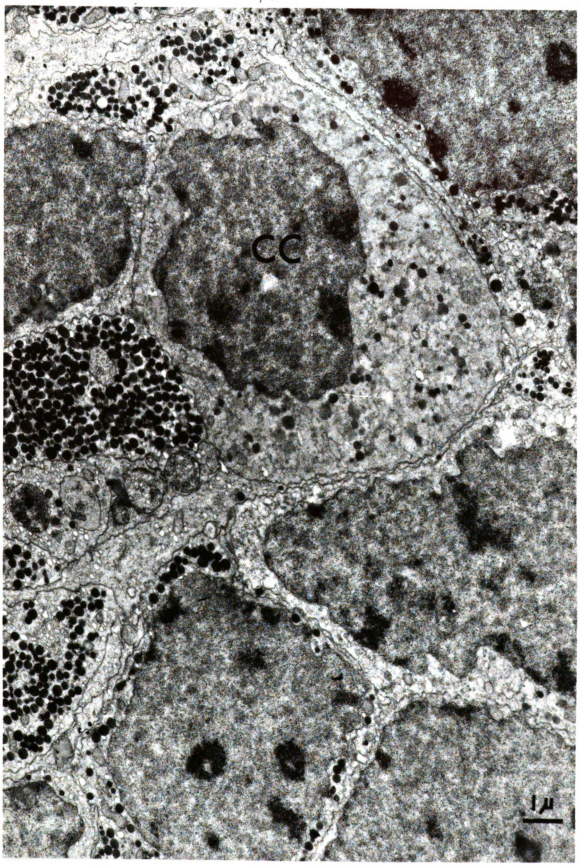


Figure 32

after transplantation (Fig. 8, 12, 35, 36). There is, however, the appearance of some unusual cells, "clear cells", in auto- and homografts which are similar to cells found in the pituitaries of two-year old, sexually active individuals and thyroxine-treated axolotls to be described in a later section (Fig. 37).

Reciprocal Xenografts, Newt to Axolotl

As a further control for the effects of the host environment on the cells of the pituitary graft, a study of reciprocally transplanted pituitaries was undertaken. Newt pituitaries transplanted to the lower jaws of hypophysectomized axolotls were fixed three weeks after transplantation and observed with the electron microscope. These grafts were found to be in an extreme state of destruction. In only one graft were recognizable pituitary cells present. The remainder of the graft was composed of cells filled with lytic bodies, blood cells and some mitotic cells (Fig. 38). The nuclei of the remaining pituitary cells help make them easily distinguishable from the other cells present in the graft since their nucleoplasm is generally darker and the chromatin material more condensed than that of the surrounding cells. The cytoplasmic contents of the remaining pituitary cells were not altered greatly, nor did the cells appear to be activated by the transplantation (Fig. 39). No preponderance of one cell type over another

Figure 38. Electron micrograph of a region of lymphocytic infiltration and destruction of a three- week newt pituitary xenograft.

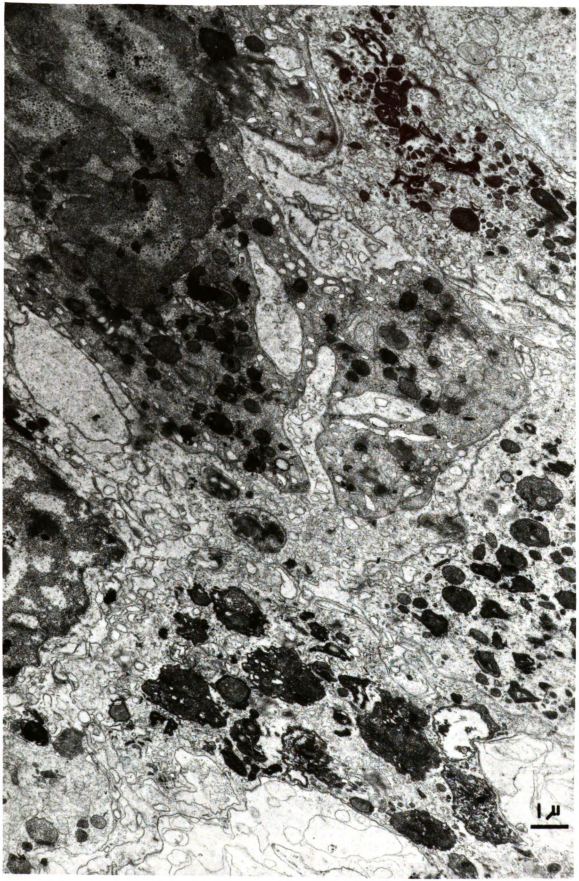


Figure 38

Figure 39. Electron micrograph of a region of a three-week newt pituitary xenograft which has not been destroyed by the axolotl host. Note absence of rough-surfaced endoplasmic reticulum in these cells.

Figure 39

could be detected in electron micrographs of these transplants. It appears that the newt grafts are being destroyed by the axolotl host by either active graft rejection, or because of the death of the graft and subsequent removal of resultant debris by the host.

In none of the transplanted pituitaries studied has the experimental intervention altered the frequency of the appearance of granule fusion with the cell surface membrane as a form of release of the cell contents described for mammals by Smith and Farquhar (1966) and by Masur (1969) and Bunt (1969) in adult urodeles. There has been no clear-cut picture of such release in the axolotl pituitary to corroborate Masur's (1969) and Cardell's (1964a) assertion that this is the primary mode of secretion in the amphibian pituitary.

Light Microscopic Evidence

Examination of differentially stained sections of paraffin embedded transplants fixed three weeks after transplantation tend to substantiate the results reported at the electron microscope level. Three week xenografts display a loss in stainability of the PAS-positive cells and a decrease in the number of alcian blue-positive cells. Orangeophils are quite numerous and easily demonstrable (Fig. 8). Auto- and homografted glands show much less degranulation (Fig. 11, 12, 13). PAS-positive and alcian blue-positive cells are more numerous than in xenografts

and acidophils are quite numerous. Four month transplants display predominantly cells which contain large PAS-positive globular inclusions. Occasionally, acidophils can be demonstrated (Fig. 10). The embedding of these glands in glycol methacrylate has limited the completeness of the staining which could be performed on these glands. Mitotic figures are obvious in all transplants, both near the surface of the graft and occasionally more internally. There appears to be an increase in the number of pars intermedia cells after transplantation, but the precise nature of this increase is as yet unknown.

Pituitaries of 2-Year Old, Sexually Mature Adults

The pituitaries of animals which are much older than those used for the transplantation experiments, and are reproductively active, show some rather interesting changes in cellular structure. Large numbers of "clear cells" similar to those appearing in auto- and homografted pituitaries are present, and in some regions of the pars distalis, there is a fusion of pituitary cells. The "clear cells" are easily recognizable, even in methylene blue stained thick sections (Fig. 40). In electron micrographs, their cytoplasm is seen to contain numerous irregularly shaped vesicles, numerous mitochondria and occasional electron dense granules (Fig. 40). The vesicles are small (approx. 100-150 mμ.) and may occur in strings or clusters. A recognizable Golgi complex is also present in some "clear cells". The few granules

Figure 40. A composite electron micrograph of clear cells and type III cells in the pars distalis of a two-year old sexually mature axolotl. The insets are light micrographs (1800X) which demonstrate the ease with which the clear cells may be seen in 1u thick sections. The cell labelled A inthe inset corresponds to A in the electron microgaph. Similarly, the cells B aand D in the insets correspond to B and D in the electron microgaph (12,000X).

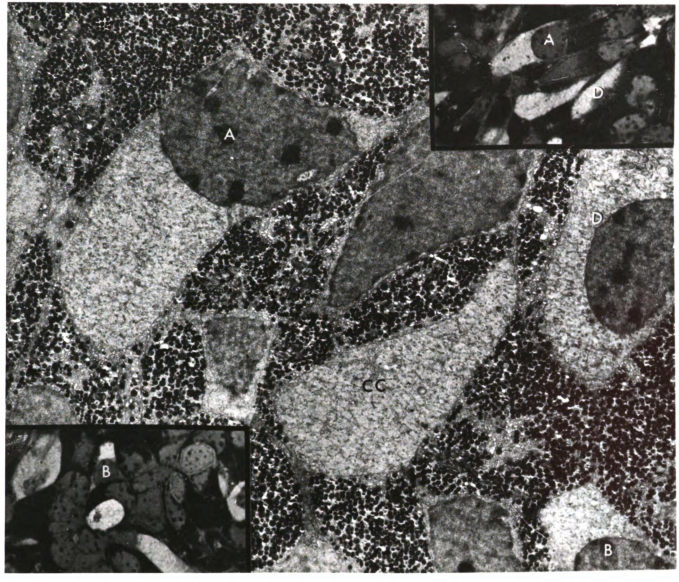


Figure 40

present in their cytoplasm appear to be in the process of breaking down. Multitubular bodies are also present quite frequently.

The rest of the cells of the older pituitaries appear similar to those of younger glands (Fig. 41, 42). Type II cells are present less frequently than in younger or thyroxine-treated glands (see below), but other cells seem to be present in normal quantities. The granules of the type III cells are slightly larger (375 mμ) than those of the younger animals and vary more in their diameters than those of the type III cells of the young animals. This probably does not represent a definite differentiation of another cell type. The Golgi complex in the type III cells is especially noticeable and granules in the process of formation may frequently be seen in this area.

In the areas around the blood vessels, there appears to be a fusion between the different cells of the pituitary. This occurs often between "clear cells" and some other cell type, but is not limited to combinations of this sort. In some areas, there are large expanses of intermingling cell contents with no discernible cell boundaries (Fig. 14, 43). There is no increase in the appearance of rough-surfaced endoplasmic reticulum in any of the cells of the pars distalis, and in fact, the "clear cells" appear to be particularly deprived of even free ribosomes.

The pars nervosa appears unchanged (Fig. 44). The cells of the pars intermedia are also essentially unchanged

Figure 41. Electron micrograph showing cell types I, II, and III in the pars distalis of a two-year old sexually mature axolotl. Arrow indicates a section through a centriole.

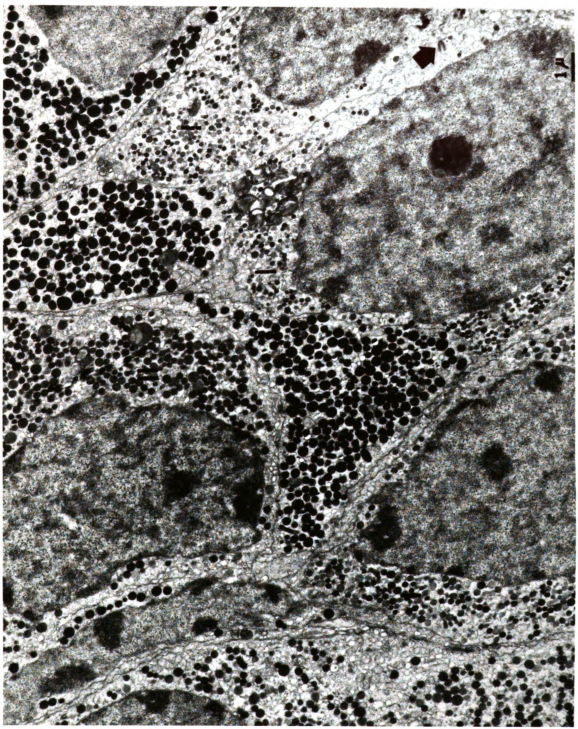


Figure 41

Figure 42. Electron micrograph illustrating the presenceof at least one, probably two, type V (stellate) cells in the pars distalis of a two-year old sexually mature axolotl.

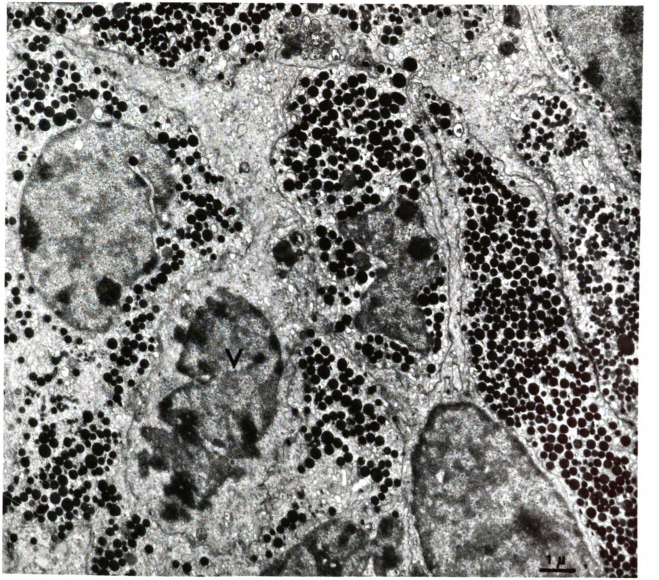


Figure 42

Figure 43. Electron micrograph of a region of cell fusion near a capillary in the pars distalis of a two-year old sexually mature axolotl.

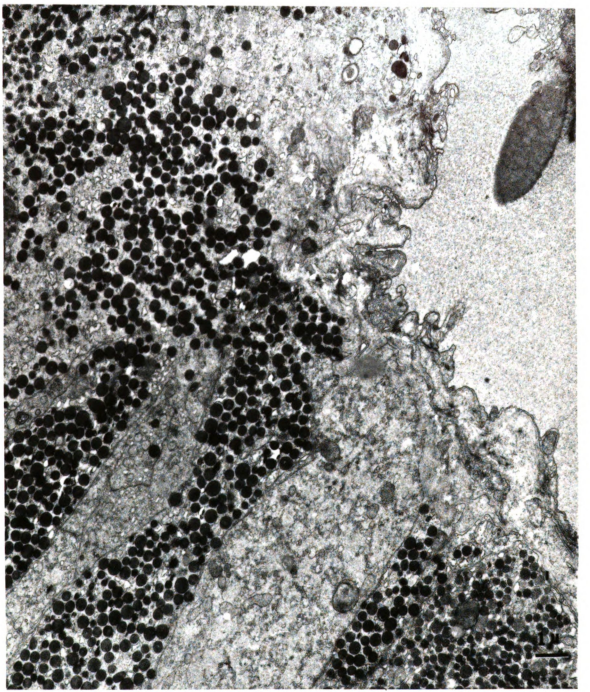


Figure 43

Figure 44. Electron micrograph of the pars nervosa of a two-year old sexually mature axolotl. The pars nervosa of axolotls appears the same throughout the developmental stages (young, mature, metamorphosed) studied.

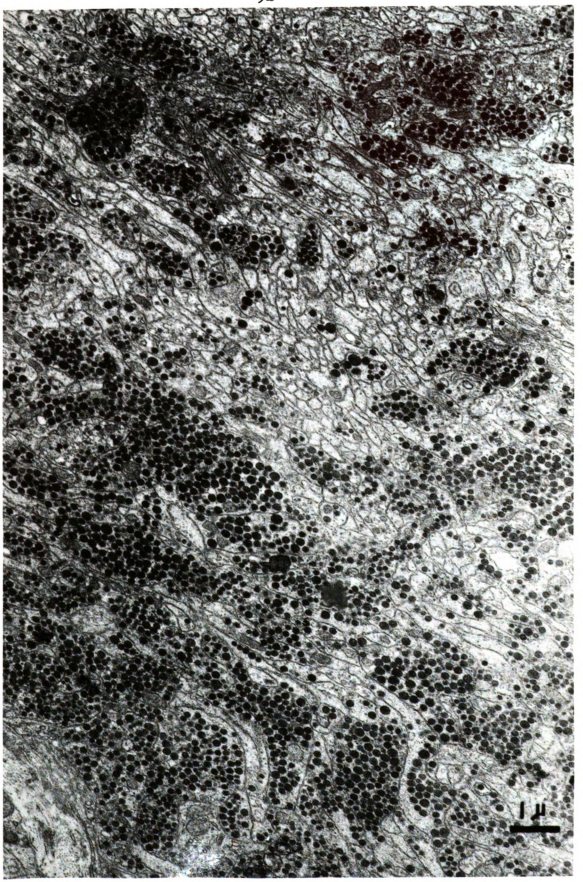


Figure 14

except for the fact that cells containing small electron dense granules appear more frequently (Fig. 45).

Pituitaries of Thyroxine-Treated Animals

Pituitaries from thyroxine-treated animals show some differences from pituitaries of young and of sexually mature animals. The type II cells are very numerous and the globular elements in these cells are very large (sometimes more than 2μ in diameter). The appearance of a complicated substructure within these globular elements is also much more evident (Fig. 46). Large amounts of rough-surfaced endoplasmic reticulum are present among the granular elements of many cells (Fig. 47) and cell fusion is very prominent, even more so than in two-year old animals (Fig. 48). Type I cells appear less frequently than in young normal or two-year old animals. In many cells, dumbbell-shaped secretory granules (Fig. 46) are found, indicating that fusion of secretory granules could be a method for enlargement of such granules after formation in the Golgi complex.

No differences could be detected in differentially stained sections of two-year old and young normal glands fixed at the same time of the year. There appears to be a regionalization of cells within the pars distalis of pituitaries of two-year old animals similar to that described for young normal animals fixed in the early winter months. No differences are detectable between

Figure 45. Pars intermedia of a two-year old sexually mature axolotl. Again, no difference can be detected in pars intermedia cells of young, mature or metarmophosed A. mexicanum.

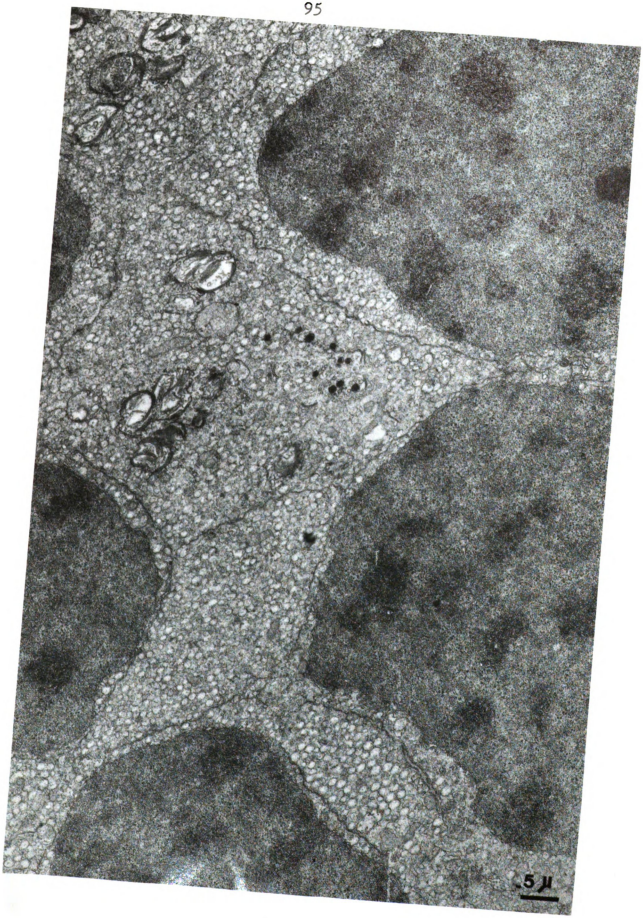


Figure 4.5

Figure 46. Electron micrograph illustrating large globular elements of a type II cell of a thyroxine treated axolotl. Note the complicated tubular substructure of the globular elements. Arrows point toward globules containing very noticeable substructure.

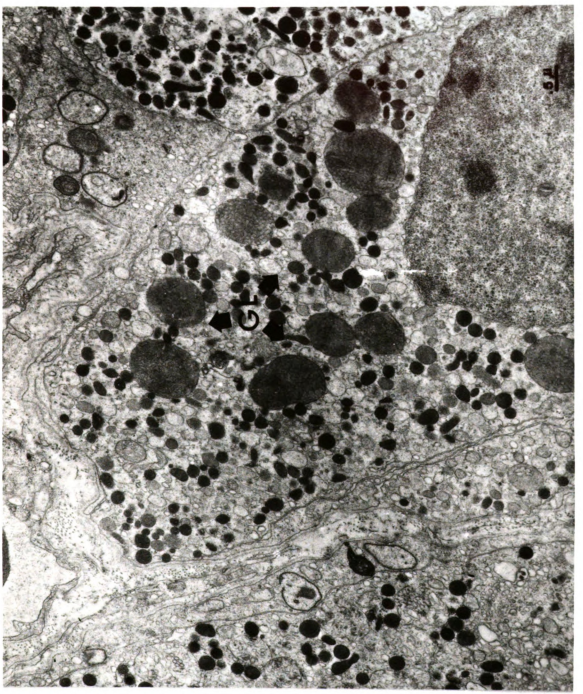


Figure 46

Figure 47. Electron micrograph of cells in the pars distalis of a thyroxine-treated animal. Two type III cells can be seen to contain large multitubular complexes. Large amounts of rough-surfaced endoplasmic reticulum (RER) can also be seen in the cell in the center of the picture.

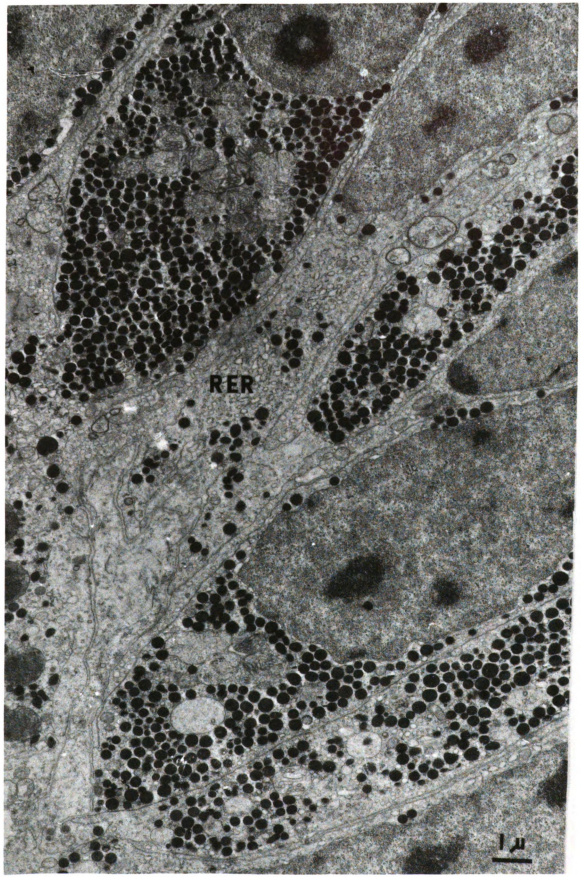

RER
1 μ

Figure 48. Electron micrograph which clearly illustrates the fusion of cells in the pars distalis of a thyroxine treated animal. F indicates an obvious fusion zone.

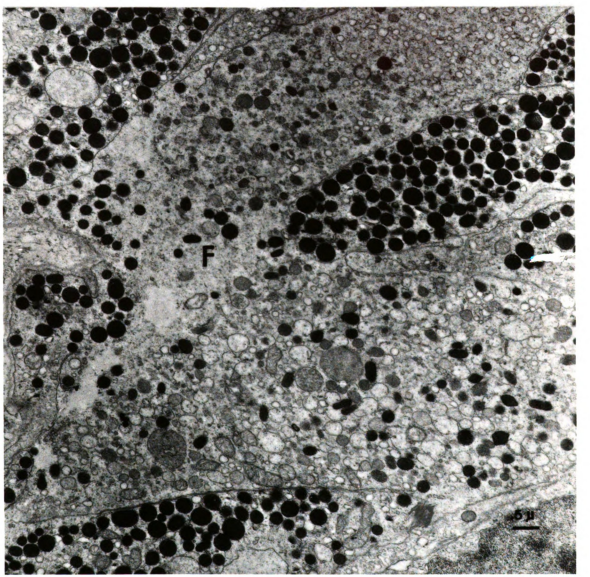


Figure 48

differentially stained sections of two-year old and thyroxine-treated pituitaries (Prahlad, personal communication). The major differences between two-year old and thyroxine-treated animals physiologically are: 1) testicular activity in thyroxine-treated animals is negligible while two-year old animals have high spermatogenic activity (Prahlad, personal communication): 2) thyroid activity in thyroxine-treated animals is considerably higher than in two-year old animals (Prahlad, 1968). The major differences between two-year old animals and young normal animals is thought to be the greater sexual activity of the two-year old animals.

Discussion

Although differential staining could not be performed reliably on epoxy embedded pituitaries, some correlation between the cells seen in differentially stained paraffin sections and those seen with the electron microscope is still possible. Due to the size of the globular inclusions in the type II cells, it is felt that they correspond to the alcian blue-positive cells containing large PAS-positive globules. The variation in electron density of the granules in these cells suggests that they would correspond to a basophilic cell at the light level. In Triturus marmoratus and some other forms, granules of acidophilic cells are considered to be heavily electron dense, while those of basophilic cells are more variable in their electron density (Doerr-Schott,

1966; van Oordt, 1968; Costoff and McShan, 1969; Seligman et al., 1968). For this reason, the type III cells described above are thought to correspond to the orange G or erythrosine-positive cells seen with the light microscope. The orange G-positive cells of the eastern newt (Diemictylus viridescens) also contain rather uniformly sized granules which are heavily electron dense (Cardell, 1964a). A precise correspondence between cell types I, IV, and V and cells seen at the light level is not yet possible. On the basis of the assertion that acidophils have extremely electron dense granules and basophils possess granules of a more variable electron density, it is expected that the type I cell is a type of basophil. Types IV and V are probably chromophobic (Cardell, 1964a, 1968, 1969).

Several cell types present in the axolotl pituitary correspond to cells described at the electron microscopic level for other urodeles (Cardell, 1964a; Doerr-Schott, 1966; Bunt, 1969). Table 1 is a brief compilation of the cytoplasmic characteristics upon which a system of classification has been formed for different cell types found in Diemictylus viridescens, Triturus marmoratus, Taricha torosa, and Ambystoma mexicanum. It can be seen that cell types II, III, and V of A. mexicanum correspond closely to cell types in the other pituitaries studied. Neither cell type I nor cell type IV has any counterpart in the other glands studied, except for a possible correlation between type I cells and the

Table I

Comparison of Amphibian Pituitary Cell Types

D. viridescens (Cardell, 1964a)	*T. marmoratus* (Doerr-Schott, 1966)	*Taricha torosa* (Bunt, 1969)	*Ambystoma mexicanum*
I 360 mu very dense	Acid. I 250-550mu very dense	I 600 mu very dense	I 260-330 mu variable density
II 220 mu very dense	Acid. II 100-180 mu very dense	II 200 mu very dense	II 330-520 mu variable density 1 u globules vesicles 130 x 330 mu
III less than 100 mu	Baso. I 160-320 mu multitub. bodies	III 100-150 mu	III 330 mu very dense
IV 120-320 mu variable density 2 u globules	Baso. II 150-300 mu variable density	IV 250 mu 2 u globules	IV vesicles 200-220 mu
V Stellate	Chromophobes	V 400 mu 2 u globules	V Stellate
		VI Stellate	

A.M.	D.V.	T.M.	T.T.
I	?	Baso. II?	?
II	IV	Baso. I	IV and V
III	I	Acid. I	II
IV	?	?	?
V	V	?	VI

basophils type II of T. marmoratus. Bunt (1969) has suggested that some cells with granules of variable electron density but with dimensions similar to those of type I cells may be a different functional state of the type I cell (Bunt 1969, Fig. 2, p. 135). Similarly, the type I cell of the axolotl might represent a different state in the activity of cell type II. However, this cell might also represent a counterpart of the aglobular basophil of D. viridescens described by Dent (1961) at the light microscope level which Cardell (1964a, b) was unable to find with the electron microscope. That this is indeed a functionally different cell type is, however, not known. The presence of only one acidophilic cell type at the light microscope level in A. mexicanum (orange G or erythrosine) supports the belief that the type III cells represent this cell at the electron microscopic level.

The functional significance of the majority of these cell types remains to be conclusively elucidated, although the results of the transplantation and the metamorphosis experiments seem to add some light as to the functional nature of some of the cell types described. The type V or stellate cell seems to be of a supportive rather than a secretory nature in the newt (Cardell, 1968, 1969). The presence of microfibrils, the length of the processes which these cells send out between other pituitary cells, and the rarity of granulation in their cytoplasm in the axolotl supports this conclusion of Cardell (1968, 1969)

for the newt.

The results of the transplantation experiments reported above parallel very closely those of Pasteels (1960), van Dongen et al., (1966), Doerr-Schott (1967) and Masur (1969). The rapid loss of granulation in the cytoplasm in the majority of the cell types in the axolotl pituitary after xenoplastic transplantation (three weeks) is somewhat faster than that reported for other pituitaries after auto- or homoplastic transplantation (Pasteels, 1960; van Dongen et al., 1966; Doerr-Schott, 1967; Masur, 1969). The results of the experiments in which axolotl pituitaries were auto-or homoplastically grafted show that there is a slower rate of degranulation when the host environment is not so drastically different from that of the donor. Assuming that the mechanisms of homo- and xenograft rejection are similar, the fact that homografted glands do not show degranulation comparable to xenografts indicates that the degranulatory effect is not completely due to rejection of the pituitary by the host. Since axolotl pituitaries do remain intact and active in the xenoplastic host (as evidenced by host survival) for up to four months, it appears that rejection processes are acting very slowly on the axolotl glands.

That the newt (Diemictylus) is more tolerant to xenografts than the axolotl is indicated by the results of several experiments. Cohen (1966a, b, c, 1968, 1969) has studied the immune response of several urodeles, particularly Diemictylus viridescens. His results

demonstrate that Diemictylus rejects Ambystoma tigrinum skin grafts less rapidly than it does homografts, and less rapidly than A. tigrinum rejects Diemictylus skin grafts (Cohen, 1969). Tassava (1969b) has reported the retention of an entire axolotl limb by a Diemictylus host for a prolonged period of time. The results of the reciprocal pituitary transplantation experiments reported above also support this conclusion, since the majority of newt pituitaries grafted to axolotls were rejected or well into the rejection process by three weeks after transplantation. Recently, it has been found that skin grafts from larval axolotls (A. mexicanum) survive longer on newt hosts than do newt skin homografts. It has also been found that newt skin grafts are rejected more rapidly by larval axolotls than the reciprocal xenoplastic grafts (Taban and Connelly, unpublished). Cohen has attributed such a phenomenon to a genetic factor in the immune system of the urodeles (Cohen, 1969). However, a second possibility must be taken into account. Skin grafts from axolotls to newts rest on a very thick collagenous layer. Pituitary grafts from axolotls to newts are surrounded by a similarly thick layer of collagen (Fig. 18). This layer is not comparably thick in newt homografts or in newt tissues grafted to axolotl hosts. It may be that this thick collagenous base serves to protect the graft in some way from destruction by the host. Cohen (1966a, b, c) has noted a thickening of

the collagenous bed in homografts which survive for prolonged periods of time, but does not suggest that this is related to the survival of the graft. In skin grafts where a thick collagenous layer is present, lymphocytic infiltration is only visible at the edge of the graft and not in the center (Taban and Connelly, unpublished).

It has been suggested (Doerr-Schott, 1967) that some of the gonadotrophic activity of transplanted glands is due to a passive release of material already present in the gland. This may also be the case for some cells in the axolotl pituitary transplanted to the newt, particularly with respect to the type I or type II cells. However, the appearance of rough-surfaced endoplasmic reticulum in xenografted pituitaries suggests that some of the cells (especially the type III cells) are being activated by the new environment. The appearance of a more striking activation of axolotl xenografts than in homo- or autografts suggests too that some factors not present in the blood of the axolotl, or present in greater quantities in the blood of the newt, cause the activation of these cells. In addition, the very rapid destruction of the newt pituitary tissue by the axolotl host might indicate that the lack of certain factors in the axolotl blood has combined with the immune response of the host to cause more rapid removal of the grafted

tissue. The slower degranualtion of auto- or homografted pituitaries of other amphibians could indicate that some maintenance factors (from the hypothalamus?) are preserving the activity of the transplant for some time. The slower degranulation of the axolotl pituitary when auto- or homografted might be interpreted as further indication that this gland depends upon hypothalamic connections less than do those of other animals.

The presence in four month transplants of cells which resemble type III cells and the predominance of type III cells in three week transplants support the idea that this cell type is the acidophilic cell seen at the light level. Acidophilic cells predominate in differentially stained sections of paraffin embedded three week transplants. Occasionally, acidophilic cells could even be demonstrated among the cells containing large PAS-positive globules in methacrylate embedded four month transplants (Fig. 10). Methylene blue stained four month transplants occasionally display metachromatic cytoplasmic contents (Fig. 9). It is quite possible that these cells are responsible for the secretion of prolactin-like hormone in normal and transplanted pituitaries, since this hormone plays an important role in the survival of hypophysectomized newts (Tassava, 1969a, b; Connelly et al., 1968a). The acidophilic cells (type I and type II) of Diemictylus show increased activity after autoplastic transplantation which is correlated with the precocious induction of the water-drive in the red eft (Masur, 1969). This

water-drive may also be induced by injection of mammalian prolactin (Grant and Grant, 1958). In other ectopically transplanted amphibian pituitaries, acidophilic cells predominate (Pasteels, 1960; van Dongen et al., 1966; Doerr-Schott, 1967), and this has been related to increased larval growth (Etkin and lehrer, 1960). That this increase in larval growth is probably due to excess prolactin-like hormone secretion has also been suggested by the results of Berman et al.(1965) and Brown and Frye (1969a, b). In ectopically transplanted or cultured mammalian pituitaries, prolactin cells (erythrosinophils) predominate in the graft in a short time (Desclin, 1963) and prolactin secretion increases (Rivera and Kahn, 1970). It is not yet possible to rule out a participation of growth hormone (STH) in survival of hypophysectomized newts (Tassava, 1969a). Therefore, one might also interpret the acidophilic or type III cell as being the site of production of an STH-like hormone, or as the site of production for both STH- and prolactin-like hormones. In some animals, STH cells are considered to be erythrosinophilic (Meites and Nicoll, 1966), but there is apparently no separation of orangeophils or erythrosinophils in the axolotl pituitary. There is also no other cell type seen at the electron microscope level which is suspected of being an acidophil other than the type III cell. It is

possible that the differences between acidophilic cells are very subtle and thus might not be satisfactorily resolved in this animal with routine light or electron microscopic methods. Attempts to identify prolactin secreting cells by using the indirect fluorescent labelled antibody technique of Weller and Conns (1951) and to test for prolactin by immunodiffusion (Crowle, 1961) with a commercially prepared antiserum against mammalian prolactin has also proved ineffective (Connelly and Taban, unpublished).

It was hoped that xenoplastic transplantation might also aid in the identification of the cells responsible for the secretion of TSH since this hormone is also believed to be an important factor in hypophysectomized newt survival (Tassava, 1969a,b). However, the rapid degranulation of all cell types of the axolotl pituitary after xenoplastic transplantation with the exception of the type III cells makes a correlation of granule content and cell function or activity with respect to TSH very difficult. Pasteels (1960) was unable to visualize TSH cells in the ectopically transplanted pituitary of Pleurodeles Waltlii. After examination of the thyroid follicles in animals with ectopic pituitary grafts, he concluded that thyroid activity was drastically reduced. However,

the fact that these animals moulted normally and regularly indicated that TSH and thyroid activity were sufficiently high to maintain this function. Similarly, ectopic pituitary autografts support moulting in metamorphosed A. mexicanum, indicating normal thyroid activity with respect to this function (Jorgensen and Larsen, 1963). In the newt Diemictylus, hypophysectomy also prevents moulting (Dent, 1966). This condition may be remedied by ectopic pituitary autografts (Dent, 1966; Tassava, 1969a) or by treatment with prolactin and thyroxine or thyroxine (Tassava, 1969a; Connelly et al, 1968a). Thus, the fact that hypophysectomized newts bearing ectopic axolotl pituitaries survive and moult as well as do animals treated with prolactin and thyroxine indicates that the graft secretes a prolactin- or STH-like hormone and TSH (see also Tassava, 1969b; Compher and Dent, 1970). The presence of numerous cells resembling type I cells in three week xenografts suggests that this cell type might be responsible for TSH secretion in the axolotl pituitary. Furthermore, the resemblance of the type I cell to the expected ultrastructure of the basophils type I of other amphibians makes a correlation even more tempting (van Oordt, 1968). The basophils type I described at the light microscope level are thought to be the source of TSH in other amphibians (van Oordt, 1968). The appearance of cells resembling type I cells in the pituitary grafts might also be

interpreted as a breakdown of granulation of other cell types, resulting in the passive release of cell contents in response to trantation. Such a resultant similarity might obscure the functional role of this cell type.

No experiments were performed with the express intent of identifying the gonadotrophic cells of A. mexicanum, but a comparison of the ultrastructure of pituitaries from 2-year old sexually active, thyroxine-treated, and young normal axolotls has indicated some possible correlations. Cell types I and II are present in about equal frequency in the pars distalis of 8-mont old larvae. These animals are not reproductively active at the time of fixation. The pars distalis of 2-year old sexually mature and reproductively active axolotls fixed during the early winter months showed a decrease in the number of type II cells. Pituitaries of two-year old animals fixed at the same time of the year but after the completion of metamorphosis induced by thyroxine showed a very large increase in the number of type II cells and an increase in the size of the globular elements within them. These animals were not sexually active at the time of fixation since their testes contained very few or no sperm (Prahlad, personal communication). The type II cells correspond to cells seen at the light microscope level described for other amphibians as basophils type II

(van Oordt, 1968) which are accepted as being the source of an FSH-like gonadotrophic hormone. The possible significance of large numbers of globular elements in cells will be discussed later.

The appearance of the "clear cell" in older and transplanted pituitaries might possibly be explained in relation to fluctuations in the numbers of cell types I and II. "Clear cells" do appear infrequently in transplanted glands, but not in glands of eight-month old larvae prior to transplantation. In transplants, and occasionally in two-year old and thyroxine-treated glands, cells which appear as intermediates between granulated and "clear cells" may be seen (Fig. 37). The "clear cells" might represent in one case (two-year old sexually active) chromophobic cells derived from gonadotrophic cells which have released their granulation in response to sexual activity. In the other case (thyroxine-treated), these "clear cells" might represent TSH cells which have released their granular contents in response to thyroxine stimulation. In transplanted glands, the "clear cells" might represent exhausted gonadotrophic cells also, since the gonadotrophic activity of ectopically transplanted pituitaries is known to be very low (Pasteels, 1960; van Dongen et al., 1966). The pituitaries of postmetamorphic animals are more active than those of the premetamorphic animals as evidenced by the large amounts of rough-surfaced endoplasmic reticulum present among the granules of many of the cells of these thyroxine

treated glands. The absence of large amounts of rough-surfaced endoplasmic reticulum in the "clear cells" argues against the theory that they are highly active. Instead, this suggests that they represent the end product of a long period of degranulation of one cell type or another which may be followed by the appearance of cellular organelles (mitochondria, RER, etc.) necessary for the synthesis of more cell product, or perhaps of even a different cell product. Finally, these cells might just represent an inactive population of cells whose role is entirely unknown. Once a cell has lost its identifying granules, its origin is difficult to determine. These cells resemble slightly the type V cells of Bufo arenarum (Iturizza, 1964, Fig. 7, p. 229) , the so-called "peculiar nongranulated cells" (PNG cells) found in the mouse pituitary (Yamada and Yamashita, 1967) and the "ordinary chromophobe" of the dog (Kagayama, 1965), the function of which is unknown.

The presence of globular elements in some cells of the pars distalis of the urodele pituitaries studied thus far is the most consistent feature of the cytoplasmic contents of these glands. These globular elements are probably lysosomes (Masur, 1969; Masur and Holtzman, 1969; Doerr-Schott, 1964, 1965; Riecken et al., 1965),

and this does argue against the theory of Cardell (1964a) that the globules and granules of the type IV cells of Diemictylus represent two different hormones secreted by the same cell at the same time. It seems more likely that the globules (lysosomes) represent a mechanism for the destruction or recycling of hormone contents which have been stored in the cell for some time without being released (Smith and Farquhar, 1966; Masur and Holtzman, 1969). The presence of multitubular bodies in many pituitary cells (in the axolotl all cell types may contain them) and the presence of globular elements with visible substructure in some of these cells supports the idea that there may be an orderly breakdown of material synthesized in the cells but not called upon for secretion (Smith and Farquhar, 1966). No evidence of granule extrusion at the cell surface, which Cardell (1964a) asserted was the probable mode of secretion in the amphibian pituitary, has been seen in the axolotl. There is, however, abundant evidence of active production of granular cell contents. Although some reports of exocytosis of granules by the pituitary cells of the salamander have appeared, this occurs only after the gland has been stimulated in some fashion (Masur, 1969; Bunt, 1969). The presence of more numerous multitubular bodies in the pituitary cells of

the axolotl and Triturus marmoratus than in Taricha or Diemictylus might indicate that the pituitaries of the former two animals are much less active than those of the latter. That the axolotl places little demand on the pituitary for hormones other than gonadotrophins is evidences by the fact that these animals will survive indefinitely (personal observation) and will even grow (Tassava, 1969b) in the hypophysectomized state. Only sexual maturity and the completion of metamorphosis induced by thyroxine are dependent upon the presence of the pituitary in the axolotl (Tassava and Connelly, unpublished observations). The low frequency with which exocytosis of granules is seen in non-stimulated newt pituitaries and the still lower frequency with which this mode of secretion is seen in the axolotl pituitary even after ectopic xenoplastic transplantation or thyroxine-induced metamorphosis suggests that some other less readily visible method of secretion is acting as the primary mode of secretion in the urodele pituitary. Furthermore, the appearance of large spaces between the cells in the pars intermedia and occasionally in the pars distalis suggests an accumulation of material (secretion?) which has passed across the cell membrane and has subsequently been removed by the procedures of preparation for electron microscopy. The

absence of granulation in intercellular spaces and blood vessels also suggests that some less visible mode of secretion is occurring in the axolotl.

Figure 49 is a diagrammatic representation of some possible modes of secretion in an axolotl pituitary cell. Dense granules formed in the Golgi complex might be extruded at the cell surface (Pathway I). This method has not been seen in the axolotl pituitary. In the case of a pars intermedia cell, a vesicle with less dense contents might fuse with the cell surface membrane but be undetectable at the time of fusion with the cell surface (See Pathway 4 below). This method of secretion has been seen in the rat (Smith and Farquhar, 1966) and in stimulated urodele pituitaries (Bunt, 1969; Masur, 1969). Some granules formed in the Golgi complex may fuse (Pathway 2) to form larger granules (such dumbbell shaped figures are often seen in the axolotl pituitary)(Fig. 16), or may break down releasing their contents into the cytoplasm before or after fusion (Pathway 3). In such a case, the contents of these granules, liberated into the cytoplasm, might be capable of diffusion across the plasma membrane and be undetected visually. Large granules or globules of very slight electron density could unite with the cell surface (Pathway 4). In this case, their contents would be released, but in a form probably indistinguishable from the extracellular matrix (see also Kurosumi, 1961).

Figure 49. A diagrammatic representation of possible modes of secretion in a cell of the axolotl pituitary. Pathway 1 indicates a possible mode of secretion which has been found in the pars distalis of the rat (Smith and Farquhar, 1966) and in stimulated pars distalis cells of urodeles (Masur, 1969; Bunt, 1969). This mode of secretion has not been seen in the axolotl pituitary. In the case of a PI cell, a vesicle with less dense contents might follow this pathway, but be undetectable at the time of fusion with the cell surface membrane. Pathway 2 represents a method for formation of larger granules. Dumbbell shaped structures (Fig.46) such as these are often encountered in pars distalis cells. Pathway 3 shows the possibility of breakdown of granules and the release of their contents into the surrounding cytoplasm. This material might then diffuse directly across the plasma membrane undetected. Pathway 4 represents the possibility of fusion with the cell surface membrane of a slightly electron dense granule or globule which would be difficult to detect, since the contents of the granule differ only slightly from those of the extracellular matrix. Pathway 5 represents a

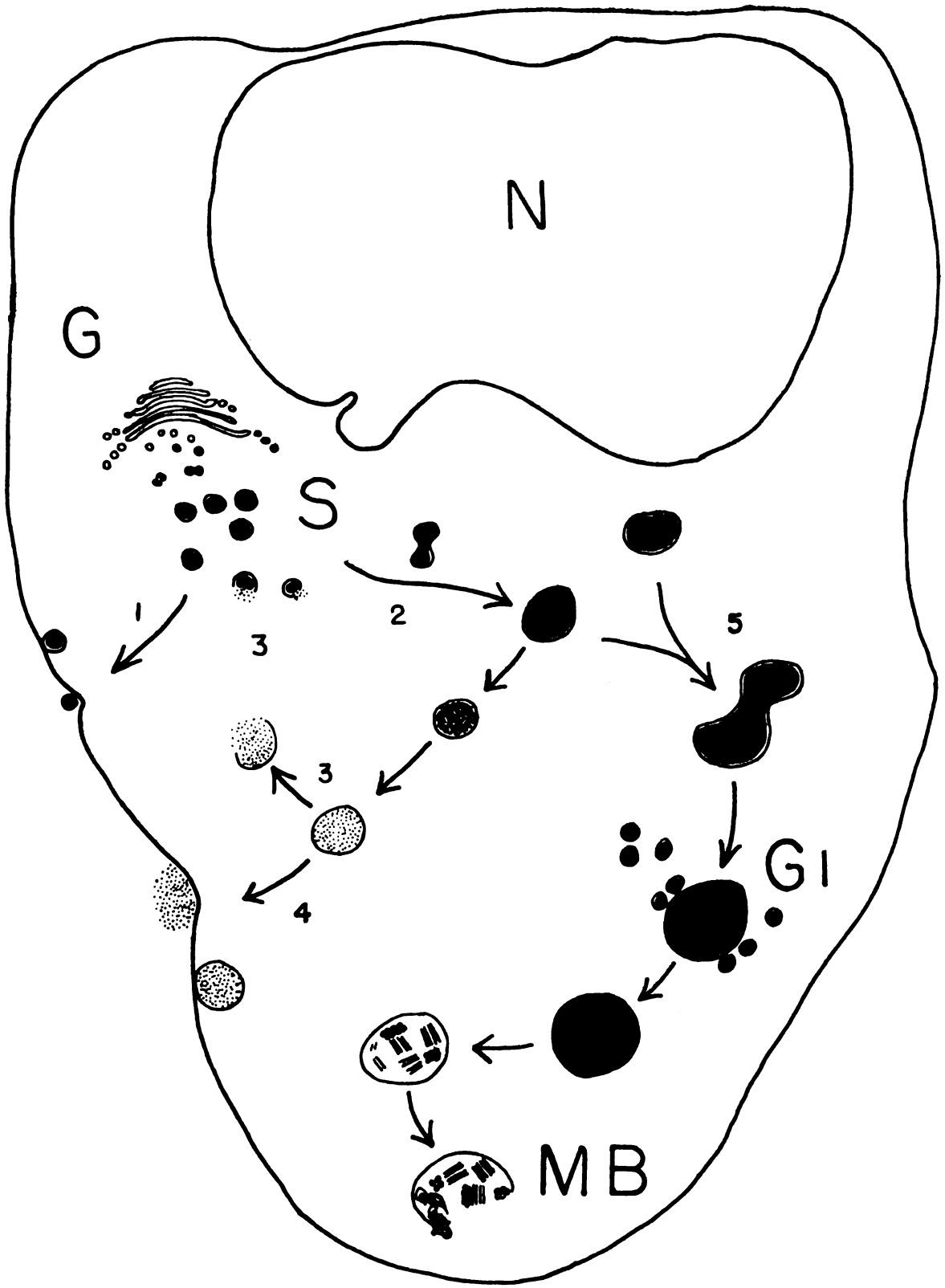


Figure 49

Figure 49 (continued)
theoretical method for the formation of multitubular structures in the axolotl pituitary. Granules and large globules (lysosomes) fuse, undergo a change in electron density, and produce material(s) which might assume a multitubular conformation upon fixation.
G, Golgi complex
S, secretory granule
GL, globule
MB, multitubular body

Please note again the substructure of globules in the type II cell in Fig. 20 and Fig. 46.

If the requirements of the animal for hormones are slight and control of pituitary cell activity is weak, some excess hormone may be produced and stored in a granular form. If after some time this material is not released, it might be broken down through lysosomal action and the breakdown products re-utilized by the cell. These breakdown products might assume the shape of multitubular bodies and intermediate structures between this form and the globular structures upon fixation (Pathway 5). Such intermediate structures are in fact frequently seen in the axolotl pituitary (Fig. 47), as are arrangements which represent fusion of smaller granules with larger lysosomal structures. That the reliance of the axolotl upon its pituitary is slight is indicated by the above-mentioned observations concerning survival and metamorphosis. The conditions suitable for a sequential breakdown mechanism thus exist in the axolotl pituitary. The question arises as to how this theory can account for the presence of multitubular bodies in all cells of the axolotl pituitary, while the globules and intermediate structures appear only in the type II cells. Although some studies indicate that granules of a certain size and electron density do possess specific hormonal properties (Costoff and McShan, 1969), this does not mean that one cell need be rigidly fixed in the type of hormone it produces. Evidence for the production of a hormone (TSH)

after thyroidectomy by cells normally considered to produce other, unrelated hormones, exists in mammals (Dingemans, 1969). Similarly, some cells may contain two different hormones at the same time in granules of similar morphology (Nakane, 1970). Multitubular bodies in a cell without lysosomal globules (such as type III cell) might be the remnants of materials of a different nature from that in the granules presently contained by the cell, which have been destroyed and replaced by a new product. Thus, the globular basophils (type II cells, type IV cells of Cardell) might represent intermediates in this process of breakdown instead of a functionally distinct cell type. The globules present in these cells probably represent the method for destruction of cell contents rather than separate hormonal contents destined for secretion. Such a mechanism of turnover under conditions of stress (or even during normal changes in the physiological state) might explain the contradiction in the results of Dent (1961b) and Cardell (1964b). The globular cells reported by Cardell (1964b) to be the probable source of thyroidectomy cells in the newt might in reality be a final step in the destruction of the contents of acidophilic cells which Dent (1961b) concluded were the source of thyroidectomy cells (see also Dingemans, 1969).

The appearance of cell fusion in pituitaries from older or thyroxine-treated animals is entirely unexpected.

Its significance in relation to hormone production or secretion is unknown at this time.

In summary, the foregoing experiments have shown that: 1) the axolotl pituitary is constructed similarly to pituitaries of other non-neotenic amphibians and appears to possess secretory capabilities similar to those of other amphibian pituitaries, thus suggesting that neoteny in this animal is not due to an anatomical deficiency in the pituitary; 2) that the axolotl pituitary reacts rapidly to transplantation to the adult newt by degranulation; 3) that newt pituitaries are not maintained in an axolotl host which may be a result of graft rejection and a lack of hypothalamic maintenance factors in the blood of the host; 4) that the type III cells may be correlated with survival in an hypophysectomized adult newt host, and are thus implicated as a possible source of prolactin-like hormone; 5) that as a result of fluctuations in gonadic activity throughout the year and after metamorphosis, the type II cells may be correlated with gonadotrophic activity; 6) that the type I cells may be correlated with TSH activity, although these cells are not present in xenoplastic grafts four months after transplantation.

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