MICROSCOPIC OBSERVATIONS OF IN VITRO CULTURES OF THE ANTERIOR PITUITARY FROM THE MALE RAT

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MICROSCOPIC OBSERVATIONS OF IN VITRO CULTURES OF THE ANTERIOR PITUITARY FROM THE MALE RAT

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INTRODUCTION

The cytologic structure of the pituitary gland has undergone considerable investigation during the last few years. During this time much attention has been given to the functional changes which develop in the cells of the pituitary in relation to different physiological states. It is well known that the microanatomy of the pituitary is very difficult to interpret in the normal animal because of the complexities which exists between the pituitary, its target organs and the hypothalamus. Therefore in an effort to eliminate some of the variables which exist in the intact animal, several procedures have been evolved to isolate the pituitary in order to better study its hormonal activity and cytology. These methods include pituitary stalk section, transplantation to the kidney capsule, transplantation to the anterior chamber of the eye, and *in vitro* culture of the pituitary.

This study is concerned with the microscopic anatomy of the rat pituitary under conditions of organ culture.

JUSTIFICATIONS FOR INVESTIGATIONS IN THIS AREA

The factors which control synthesis and release of hormones by the endocrine organs, particularly the pituitary, are not well understood at the present time and any information in this area should be very useful as a basis for future studies. Physiologic studies of endocrine organs have advanced far ahead of cytologic studies,

and this is particularly true in domestic animals. Since the rat is primarily a laboratory animal, most of the work has been done on this species. A basic understanding of pituitary function in this species should be helpful for studies on other species.

GROSS ANATOMY OF THE RAT PITUITARY

The hypophysis or pituitary gland is located beneath the third ventricle of the brain, where it is attached by the hypophyseal stalk. In most animals it is situated in the depression of the sphenoid bone known as the sella turcica. However in the rat the pituitary rests on the surface of the sphenoid bone, because of the absence of the sella turcica. Here it is covered by the dura mater.

In most animals the pituitary is an ovoid gland, but in the rat it is wedge shaped, elongated laterally, and the pars distalis constitutes the major portion of the gland (Purves and Griesbach, 1951).

The gland is divided into two major components: (1) the neurohypophysis (lobus nervosa) which is further divided into the neural stalk (infundibulum) composed of the median eminence, infundibular stem, and the infundibular process (neural lobe); and (2) the adenohypophysis (lobus glandularis), which is further divided into the pars tuberalis, pars intermedia, and pars distalis. Two other terms, which should be defined are (1) the anterior lobe, which includes the pars distalis only, and (2) the posterior lobe which consists of the infundibular process and the pars intermedia (Maximow and Bloom, 1952; Ham and Leeson, 1961).

REVIEW OF LITERATURE

I. Classification of Cell Types.

A. Acidophils-basophils.

Cells of the anterior lobe of the pituitary were first classified and identified by staining reactions utilizing the trichrome stains (Schoneman, 1892; Severinghaus, 1932). Those cells which stain with acid fuchsin, erythrosin, carmine, or orange G are designated as acidophilic cells, while those cells staining with aniline blue or light green were designated basophilic cells. The disadvantage of this system was that the so called acidophilic cells stain with acid dyes as well as basic dyes, and the basophilic cells stain with either acid or basic dyes, all depending on the concentration of the dye and the pH of the staining solution (Peterson and Weiss, 1955; Singer, 1952). This terminology was further extended. Those cells which stain with erythrosin were termed erythrosinophilic cells, and those staining with carmine are termed carminophilic; the same was true for the orange G staining cells. The major disadvantage of this system in that there appears to be a considerable difference in the staining reactions between functionally similar cells in different species (Purves, 1961). The use of the terms, "acidophils" and "basophils" have become so deeply engrained in the literature that it is doubtful that they will ever be eliminated completely.

B. Greek Letter Classification.

The second method of classification was based on the use of the Greek alphabet to designate different functionally active cells. This method was first used by Bailey and Davidoff (1925) but was developed to its fullest extent by Romeis (1940) in classifying human pituitary cell types. By attempting to apply this classification to different animal species, considerable confusion has resulted. The problem is similar to the acidophil-basophil classification in that the cells are identified by staining reactions and the reactions vary between different species. However, even with the handicaps of this method, it was a monumental contribution, since prior to this time no one had made a concentrated effort to relate cell types to their functions. Halmi (1950) in his identification of two types of basophils in the rat used delta to designate the gonadotropic cell and beta to designate the thyrotropic cell. The only other similar attempt was by Goldberg and Chalkoff (1952) to identify the pituitary cells of the dog using Roman numerals. This method was not generally adopted except that the terms alpha and beta are sometimes used to designate the acidophilic and basophilic cell types.

C. Correlation with Hormones.

The third method of classification has developed over many years, and is the result of hormone assays and attempts to correlate functional changes with specific cells in the anterior pituitary. Among these changes in cytology might be mentioned the absence of acidophilic cells in dwarf mice or the hyperplasia of the acidophils in human giants or in cases of acromegaly. Here these cells have been correlated with the presence or absence of growth, and are thus

correlated with the elaboration of a growth hormone and have been designated "somatotropic hormone cells" (STH) (Turner, 1960). Similarly castration of rats produce hypertrophic basophilic cells and hence have been associated with the gonadotropic hormone cells (FSH and LH) (Stein, 1932-33). In addition, thyroidectomy produces hypertrophic basophilic cells, designated thyrotropic hormone producers or TSH cells (Farquhar and Rinehart, 1954). Lactation and gestation also produces alterations in the granules of some acidophilic cells, and these were designated luteotropic or prolactin producers (LTH) (Hymer et al., 1961). The adrenocorticotropic hormone producing cell has been a matter of controversy. In the rat, it may be produced by a chromophobe (Siperstein, 1963). From these investigations have evolved the classification of cells as STH, LTH, FSH, LH, TSH, and ACTH producing cells.

II. Methods of Demonstrating Specific Cell Types

A. Periodic Acid Schiff Reaction.

The periodic acid Schiff reagent (PAS) will demonstrate carbohydrate complexes, and in particular glycoproteins (McManus, 1946; Pearse, 1952) such as those contained in the granules and vesicles of the basophilic cells (Purves and Greisbach, 1951). This reaction is not entirely specific for basophils, for a weak reaction will occur in the acidophils, but this is generally masked by employing a counterstain which will overshadow it. The specificity of the PAS stain lies in the fact that the basophils have more glycoprotein than the acidophils, and are therefore much more prominent.

B. Aldehyde Fuchsin Stain

The aldehyde fuchsin stain was originally developed by Gomori

(1950) as an elastic tissue stain and was thereafter utilized by Halmi (1952) to differentiate thyrotropic hormone cells from the gonadotropic hormone cells in the rat. It has since been modified by Elftman (1959c) so that it is more reliable. This procedure stains TSH producing cells specifically in the rat (Purves, 1961).

C. Extraction Procedures

The use of the PAS and aldehyde fuchsin stains will show the difference between two types of basophils, the gonadotrophs and the thyrotroph. However it is well known from biological assay that there are two types of gonadotropic hormones produced by the anterior lobe of the pituitary (Barrnett et al., 1955). These two cells can not be distinguished by staining, but they have been identified by extraction procedures prior to fixation of the tissues for sectioning and staining. It has been shown that 2.5% trichloracetic acid will remove all of the staining ability of the basophils except the one responsible for the production of luteinizing hormone. This has been confirmed by hormone assay after extraction as well as staining procedures (Barrnett et al., 1956; Barrnett, 1958). Therefore by these procedures, three basophilic cell types can be differentiated and correlated with individual hormones.

D. Trichrome Stains

The acidophilic cells (LTH and STH) can readily be demonstrated and distinguished in some species by the trichrome stains (Dawson and Friedgood, 1938-39). Landing (1954) compiled a very comprehensive review on the multitudes of staining methods that have been tried in differentiating cell types of the anterior lobe of the pituitary. The demonstration of these cells is not difficult in most species, but the differentiation of cells can be more easily accomplished in

some species, i.e. dog and cat (Dawson, 1946; Dawson and Friedgood, 1938-39). The rat pituitary is not easily differentiated, however, Sanders and Rennels (1959) reported that two types of acidophils can be distinguished by a modified azan stain. This stain has not proven entirely reliable by other investigators. Recently, Herlant (1960) and Pasteels (1963a) described other modifications for differentiation of acidophils in the rat pituitary.

E. Fluorescent Antibody Technics

By the process of elimination only one source of hormone is unaccounted for, namely the adrenocorticotropic hormone. This hormone is evidently produced by a different cell type in different species (Purves, 1961). In the rat some attempts have been made to use fluorescent antibodies to determine the site of its production. The results, though not entirely accepted, indicate that the basophils are the probable site of production (Marshall, 1951).

Similar studies on the site of prolactin production have shown that an acidophilic cell is responsible for its elaboration, and agreed with the results of hormone assay and staining procedures (Emmart et al., 1963).

F. Radioautographic Procedures

This procedure has been used to a very limited extent. Siperstein (1963) has shown that the rate of uptake of tritiated glycine is greatly increased in a chromophobic cell type after an animal has been adrenalectomized. It is her opinion that a chromophobic cell type is the source of ACTH in the rat pituitary.

III. Present Knowledge of the Microanatomy of the Rat Pituitary

Any description of these cells is only partially correct, for they

are in a constant state of transformation. The descriptions given here apply generally to the phase in which granules are present. It must be realized that the same cell may also exist in a non-granular form.

A. Growth Hormone Cells

These cells have an affinity for all acid dyes, and the granules are the intracytoplasmic components responsible for this affinity. The cells are considered to be columnar in shape and located along the blood sinusoids of the pituitary. The nucleus is ovoid in shape and contains 1-2 nucleoli. Electron microscopy has revealed granules in the size range of 350-500 mu. The granules vary in number with the secretory phase of the cell. The Golgi apparatus has a condensed appearance and may show the "negative Golgi" image during stages of secretion.

B. Luteotropic or Prolactin Cells

These cells have a similar staining affinity to the STH cell but are more ovoid in shape and are located on the interior of the cords that make up the architecture of the anterior lobe. The nuclei and nucleoli are again similar to the STH cell. One prominent difference is the size of the cytoplasmic granules. In the LTH cell the granules are 600 mu in size and are considerably larger and more prominent than the granules of the STH cells. The Golgi apparatus is prominent and shows the "negative Golgi" image during the secretory stages.

C. Follicle Stimulating Hormone Cell

This is one of the cells of the basophilic component and therefore has an affinity for the basic dyes, and is PAS positive. These cells are round and are located on the sinusoids together with the

STH cells. These cells are nearly twice the size of those of the acidophilic group. The nucleus is round, centrally located and contains one to several nucleoli. The granules are considerably smaller in size (140-200 mu), below the resolution of the light microscope and therefore give the cell a more homogenous appearance. The granule size and number again varies with the phase of secretion of the cell. The Golgi apparatus is prominent and shows a very characteristic "negative Golgi" image during active secretion. This cell also has large vacuoles which vary with the stage of secretion, forming a large vacuole after castration and giving rise to the term "signet ring cell".

D. Luteinizing Hormone Cell

This cell is the second member of the basophilic group, and also the other member of the gonadotropic cells, of which there are two, FSH and LH. It has the same staining characteristics as the FSH cell, and can not be distinguished from the LH cell. This cell is round and is located in the cords of cells away from the sinusoids. The granules are of the same magnitude as the FSH cell, 100-200 mu. One characteristic feature of this cell is an elongated nucleus which is folded in the middle, giving it a kidney-bean shape. The nucleoli are not always visible. The Golgi apparatus is prominent and forms a "negative Golgi" image during secretion. Vesicles are present and irregular in shape.

E. Thyrotrophic Cells or Thyroid Stimulating Hormone Cells

This is the third member of the basophilic group with the same staining characteristics as the other two. However in addition, the granules of this cell stain specifically with aldehyde fuchsin. This cell is angular in shape and has a tendency to be concentrated

in the center of the gland. The granules are small, 140 mu, and are not distinguishable with the light microscope. The nucleus is round and centrally located with one to several nucleoli. The Golgi apparatus is poorly developed.

F. Adrenocorticotrophic Hormone Producing Cell

This cell is generally considered to be a chromophobic cell because it does not stain. It is an irregular cell which is located on the interior of the cords of cells, but sends small projections or microvilli out to contact the sinusoids. The nucleus is generally much larger than the other cells, and is hyperchromatic. It contains one to two nucleoli. There are few granules and these are 100-150 mu in size. A Golgi apparatus has not been demonstrated.

G. Chromophobic Cell

This cell, if it is a separate entity, has been described morphologically. It may be that it is just one of the chromophilic cell types in a degranulated phase. However for purposes of classification I will include its morphological description. This cell has no staining reaction, it is angular or elongated in shape, the nucleus is ovoid and hyperchromatic. No granules have been reported. There is only a slight rim of cytoplasm and the Golgi apparatus is poorly developed. This cell has no particular location but may be found anywhere throughout the anterior lobe.

For purposes of clarification and simplicity, Table I includes the morphologic description of the cells of the normal anterior lobe of the rat pituitary (Farquhar and Rinehart, 1954; Herlant, 1964; Hymer, et al., 1961; Purves and Greisbach, 1952; Purves, 1961; Rinehart and Farquhar, 1953).

TABLE 1

CELL TYPES OF THE NORMAL PITUITARY

Cell Type	Stain Affinity	Granules	Shape of Cell	Shape of Nucleus	Number of Nucleoli	Golgi Net	Location
STH	Acid Positive	300-500 mu	Columnar	Ovoid	1-2	Condensed	Along the sinusoids
LTH	Same as STH	600 mu	Ovoid	Ovoid	Not reported	Prominent	On interior of cords
ACTH	Negative *HC nucleus	100-150 mu	Irregular	Large Oval	1-2	Not seen	Interior of cords but contact sinusoids
FSH	Basic & PAS pos.	140-200 mu	Round	Round	1- several	Prominent	On sinusoids
LH	Same as FSH	100-200 mu	Round	Elongated & folded	Not always visible	Prominent	Interior of cords
TSH	Same as FSH and **A.F. pos.	140 mu	Angular	Round	1- several	Poorly developed	In center of gland
CHROMOPHOBES	Negative *HC nucleus	Absent	Angular or elongated	Ovoid	Not reported	Poorly developed	None specifically

*HC = Hyperchromatic nucleus

**A.F. = Aldehyde Fuchsin positive

IV. Cell Counts

The determination of the relative percentages of cell types can only be established by counting many microscopic fields from a histologic section. This must be carried out in such a manner as to obtain a representative sample of the pituitary gland. Some investigators have published norms for the rat pituitary (Wolfe and Cleveland, 1933; Wolfe, 1935; Baker and Everett, 1947).

I believe these are only of value from a comparative aspect. Norms must be determined by each investigator to have an accurate comparison of his individual circumstances. This is necessary because there are many factors which influence cell counts. The sex of the animal (Golden, 1959), and the estrus cycle (Wolfe and Cleveland, 1933) will produce noticeable differences. Also age is an important consideration, since for example, immature rats show a predominance of acidophils and mature rats may show a decreased number of basophils (Wolfe, 1935). Another factor is species, even differences have been recognized between different breeds. This is especially true of dogs (Stockard, 1941). Another major factor is the trophic action which exists between the pituitary and its target organs; in other words the metabolic activity of the animal greatly effects the relative numbers of cell types. All of these factors must be considered when cell counts are to be interpreted.

V. The Control of Variables in the Study of Physiology and Cytology of the Pituitary

Many variables can readily be controlled, including, sex differences, age, species, and even metabolic activity to a great extent. However, it is obvious that the trophic relationship between the pituitary and its target organs can not be controlled, nor can

the mechanism between the hypothalamus and the pituitary in the living animal be controlled. As a result several methods have been developed in an attempt to control some of these factors both in the living animal and in an artificial environment. Pituitary stalk section separates the pituitary from hypothalamic control while leaving the gland in the live animal (Desclin, 1949). Pituitary transplants to the kidney capsule (Rennels, 1962), anterior chamber of the eye (Martini et al., 1959) or subcutaneous sites (Bardin and Liebelt, 1960) are of a definite value since the pituitary remains in a live animal. Some of the disadvantages are that there is considerable infiltration of the gland by fibrous tissue and some growth of new pituitary cells, making the cytology difficult to interpret. The in vitro culture method has several advantages since the secretions of the gland can be more easily analyzed, and the environment can be controlled much easier (Meites et al., 1961). Also the cytology is not influenced by growth changes or infiltration by connective tissue.

It is of particular interest that in any of these methods the pituitary has demonstrated a definite need for hypothalamic stimulation for the elaboration of five (LH, FSH, TSH, ACTH, and STH) of the six hormones released (Harris, 1955).

VI. Cytology of Pituitary Transplants

In the kidney capsule transplants the acidophilic cells are the most numerous cell type and these are reported to have undergone extensive degranulation. Rennels (1962) has shown by light and electron microscopy that the LTH cells are the most prominent cell type. In this cell the granules are large and irregular and

there is an enlarged Golgi, nucleus, and nucleolus. The STH cell decreased in numbers, and in those that are seen the cells as well as the granules are small. The basophils gradually decrease in number and generally show a degenerating condition that are absent after 18 days. The TSH cells are present at 16-17 days in fairly normal conditions but absent by 18 days. The chromophobes are a predominant cell type and the ACTH cell has not been reported. The same general picture is true for the stalk sectioned pituitaries, anterior chamber and subcutaneous transplants. The cytology of the pituitary transplants to the kidney capsule site is summarized in Table 2 (Cowie et al., 1960; Desclin, 1949; Everett, 1956; Martini et al., 1959; Nikitovitch-Winer and Everett, 1959; Quilligin and Rothchild, 1960; Rennels, 1962; Sanders and Rennels, 1959).

VII. Cytology of the Pituitary in an In Vitro Culture

The information available on the cytology of the anterior pituitary in an in vitro culture is very sparce, although similarity to that of the transplants is reported. Acidophils are the most numerous cell type and are generally reported to be degranulated. However, the reports vary with different investigators. The basophils are very small or absent. The chromophobes are abundant in number and large at the periphery.

TABLE 2

CELL TYPES IN KIDNEY CAPSULE AUTOGRAPH

STH	LTH	FSH & LH	TSH	ACTH	Chromophobes	Reference
Rare	Many, 2X diameter enlarged nucleoli Giant Golgi	Absent after 12 days	Absent after 18 days	NR	Many	Sanders and Rennels (1959)
NR	NR	Many after 1st week Small 16-17 days	Many after 1st week Small 16-17 days	NR	NR	Nikotovitch-Winer and Everett (1959)
Increased degranulation of acidophils		Absent	NR	NR	NR	Desclin (1949)
Increased degranulation of acidophils		Absent	NR	NR	NR	Everett (1956)
Predominantly acidophils		Absent	NR	NR	NR	Quilligan & Rothchild (1960)
Few and not densely granulated		Decreased Irregular shape	Decreased	NR	Large, vesicular nuclei	Martini et al. (1959)
Few acidophils		Absent	NR	NR	Many present	Cowie et al. (1960)
Few small cells, granules small, Golgi less prominent	Many cells large granules	Few, clumped cytoplasm	Few, but normal	Microvilli extending to extracellular space	Few, small no granules	Rennels (1962)

NR = Not Reported

Pasteels and Mulnard (1961), and Pasteels (1963a, 1963b) reported that the acidophilic cells can be readily identified from tissue culture explants. They have cultured pituitaries for as long as 6 weeks but report that after a few days the chromophilic cells degenerate very rapidly, except for the prolactin cell which multiplies and retains its secretory activity. The cytology of the pituitary in culture is summarized in Table 3 (Pasteels and Mulnard, 1961; Pasteels, 1963a, 1963b; Martinovitch et al., 1962; Martinovitch, 1950, 1954; Meites et al., 1961; Schaberg and de Groat, 1958).

TABLE 3

CELL TYPES IN THE ANTERIOR PITUITARY DURING *IN VITRO* CULTURE

STH	FSH & LH	TSH	ACTH	Chromophobes	References
Degranulation & degeneration	NR	NR	NR	Abundant at periphery	Martinovitch (1950)
Predominately acidophils	Few atrophic basophils		NR	NR	Martinovitch (1954)
Thickly granulated acidophils	Few basophils seen		NR	NR	Martinovitch *et al.* (1962)
Predominately acidophils	Few atrophic basophils		NR	NR	Meites *et al.* (1961)
Degenerated except for LTH which increases	Degenerate rapidly		NR	NR	Pasteels *et al.* (1961, 1963a, 1963b)
Few acidophils	Small non-granulated basophils.		NR	Majority of cells seen	Schaberg and de Groat (1958)

NR = Not Reported

MATERIALS & METHODS

I. Animals

Male rats (60-90 days of age) of the Carworth CFN strain were used in this experiment. The animals were housed in an air conditioned room under uniform temperature and lighting (76 $\pm$ 1°F and fourteen hours light daily). Four to eight-week-old White King squabs were used as assay animals in the determination of the amount of prolactin released into the culture medium.

II. Culture Methods

Disposable sterile Petri dishes were used to hold the culture medium. These were small plastic dishes having a diameter of 3.5 cm. and a height of 1 cm. Stainless steel rectangular platforms were used in the plastic dishes. These were cut from sheets of stainless steel mesh and were further formed by bending a stainless steel piece 1 cm. X 2.5 cm. at each end to form a platform 1 cm. wide, 1.8 cm. long and 4 mm. high. Then these were placed inside the plastic Petri dishes with 3 ml. of medium where they were used to support the tissue explants. Prior to placing the explants on the platforms, the explants were placed on a sterile, washed, strip of lens paper (1 cm. X 2 cm.). The ends of the lens paper extended over the sides of the platform into the medium where they acted as wicks. This procedure enabled the tissues to obtain nutrients from the culture medium and allowed them to be maintained in a moist condition while

providing optimal conditions for respiratory exchange. The Petri dish is closed by its lid but an air tight seal is not obtained (providing the lip of the lower dish does not become wet) and the atmosphere inside the dish can exchange readily with the external gases in the plastic chamber. This chamber was constructed from plexiglass for the primary purpose of gassing the cultures. This chamber measured 12.5 inches across the front, 9.5 inches in height and was 12 inches deep. It was equipped with a door which was held in place with screw clamps. The door had weatherstripping insulation around the edges to provide an airtight seal. The plexiglass used for this chamber was ¼ inch thick. The shelves inside the chamber were constructed from 1/8 inch plexiglass and were perforated with numerous ¼ inch diameter holes to facilitate mixing of the gases. The shelves were supported inside the chamber by strips of ¼ x ¼ inch plexiglass which were attached to the inside walls. They could therefore be removed and replaced by sliding them along the strip supports. Each of the shelves in this chamber could accommodate up to 30 of the small Petri dishes. The chamber was equipped with 2 gas inlets and 2 outlets. One of the outlets was attached to a mercury manometer and when the chamber was in operation the gas inflow and outflow was adjusted to give an intrachamber pressure of 10 mm. Hg. above atmospheric pressure. All cultures were incubated at 35 $\pm$ 1°C, and gassed with 95% O_2 and 5%CO_2 (Nicoll, 1962).

Standard recommendations were followed in washing and sterilizing all glassware and instruments (Merchant et al., 1960). The water used for the final rinses of the washed glassware and instruments was purified by distillation, passed through a deionizing resin column and de-organified by percolation through another resin column.

For preparation of the culture medium this water was purified further by a single distillation from glass. The stainless steel platforms were cleaned by soaking in 1/2 concentrated hydrochloric acid for 15 minutes and washed in running tap water for 30 minutes. The rafts were then rinsed 4 times in purified water, immersed in absolute ethanol for 30 minutes, immersed in diethyl ether for an additional 30 minutes, and finally allowed to dry in air. The platforms were then placed in a Petri dish and sterilized. All materials were sterilized by autoclaving, except those which would be destroyed by the high temperature. Labile materials were sterilized by passing them through a Millipore bacteriological filter* with a pore size of 0.45u.

III. The Culture Medium

Synthetic medium "199" was used exclusively for these studies. This medium contains all of the known essential metabolites for cellular nutrition and maintenance in vitro, but usually does not support cellular proliferation (Merchant et al., 1960). The 199 medium was obtained from Difco or Microbiological Associates, Inc.** in bottles containing 100 ml. of sterile, 10-fold concentrated stock solution. The ingredients used for the preparation of 100 ml. are shown on the next page.

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*Bedford, Massachusetts

**Bethesda, Maryland

Preparation of 100 ml. of "199" Culture Medium

Component	Volume
199 10X Stock	10 ml.
Antibiotic stock (5.0 mg. Streptomycin and 2,500 U Penicillin per ml.)	2 ml.
5.6% NaHCO3	6 ml.
Purified water	82 ml.

The final medium therefore had a concentration of 0.1 mg. Streptomycin sulfate and 50 U Penicillin per ml. .

The NaHCO3 and antibiotic stocks were sterilized by Millipore filtration and stored in a sterile plastic 25 ml. bottle until use. The antibiotic stock was frozen at 20°C and the bicarbonate solution was stored in a refrigerator at 4°C.

The Streptomycin sulfate and Penicillin G potassium used in the culture medium were obtained from Nutritional Biochemical Corporation.*

IV. Culture Procedure

The animals were decapitated and their heads were placed for a few seconds into a beaker containing 70% ethanol. This moistens the hair of the heads and reduces the chances of contamination of the cultures. The skull was exposed by cutting the skin with scissors and the top of the skull was removed with bone cutting scissors. The scissors were pre-soaked in 70% ethanol before use. The pituitary gland was then exposed by reflecting the brain caudally with a sterile forceps. A second pair of sterile forceps was then used to separate and discard the posterior lobe of the pituitary and the anterior lobe was removed. The gland was then placed in a sterile plastic

*Chicago, Illinois

10 cm. x 1.5 cm. Petri dish containing a few drops of "199" medium. Each anterior was halved longitudinally and each half cut into 3 explants of about 2 mm. diameter. Scalpels with #11 disposable blades (Crescent Mfg. Co.*) were used for the dissection. The plastic Petri dishes were found to be very satisfactory for dissecting the pituitary glands. The explants were then placed on the lens paper rectangles and transferred to the stainless steel stand inside a culture dish into which medium had previously been added. The culture dishes containing the explants were transferred to the gassing chamber inside the incubator. The entire process from removal of the glands to placing the tissues in the culture dishes was performed inside a plexiglass hood. Sterility inside the hood was maintained by a 15 watt ultraviolet germicidal lamp which was left on when the hood was not in use. Immediately before use the inside of the hood was washed with 70% ethanol. The medium from the cultures was changed every 3 days, and 3 ml. of medium was used in each dish. The medium from each culture dish was collected separately and stored in a freezer until used for assay.

V. Assay Procedure

The culture medium was assayed for prolactin activity by the pigeon crop method of Lyons (1937) as modified by Reece and Turner (1937). The samples were injected daily in 0.1 ml. volumes for 4 days in the same area subcutaneously over the crop gland. At the end of 4 days the birds were sacrificed and opened so that the area of proliferation could be measured and recorded. The area of proliferation

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*Fremont, Ohio

was measured by the use of wire loops of varying diameter, equivalent to a designated number of Reece-Turner units.

VI. Histological Procedure

Four glands were removed on each successive day and 1/2 of each gland was placed in Bouin's fluid and the other 1/2 in formol-sublimate.

A. Masson's trichrome and aldehyde fuchsin combined procedures are used to demonstrate the acidophilic cells and the thyrotropic cells. (Baker, 1962).

B. Periodic Acid Schiff and Orange G Method was used to demonstrate the basophilic cell types. This method is also the most useful in making cell counts (Purves and Griesbach, 1951).

Both of these procedures are outlined in detail in the Appendix.

VII. Techniques for Cell Counting

A. Procedure

1. Cell counts were determined by the "random shot" method of Chalkley, (1943). In this procedure a grid containing 25 squares was placed in a 10X ocular. The oil immersion or 97X objective was utilized in counting, providing a magnification of 970X.

2. In this method every cell which falls under a crossed line was counted, or any designated number of cross hairs may be counted. In my procedure I used only the four corner cross hairs. The procedure is to begin on the left hand side of a microscopic section and to systematically proceed through every field. Every cell

which falls under the four corner cross hairs is recorded until 1,000 cells are obtained.

3. This procedure has been used very effectively by Bakke et al. (1964) in a study on the TSH cell morphology and TSH content in the human adenohypophysis.

4. In my work only 4 cell types were recorded: acidophils, basophils, chromophobes, and degenerating cells. There was no attempt made to differentiate the acidophils and basophils as to specific cell type.

B. Criteria for Degenerating Cells

1. Pyknosis of the nuclei, seen as a decrease in size and the appearance of a small round black or dark homogenous nuclei.

2. Karyorrhexis or fragmentation of the nucleus.

3. Karyolysis or dissolution of the nuclear material.

4. Loss of the cell outline.

5. Loss of differential staining characteristics. Most degenerating cells become acidophilic in staining affinity (Smith and Jones, 1961).

RESULTS

I. Viability of Cultures

Since the anterior pituitary is removed intact from a normal animal, the percentage of viable cells is assumed to be 100%. This is essentially true, although it is obvious that there is a constant loss of cells and a regeneration of new ones. In this investigation the control animals were considered to possess 100% viable pituitary cells.

Day One

After 24 hours in an organ culture system the average viable cell count is 72.72%, or a loss of 27.28%.

Day Three

After 72 hours in culture the cell counts showed a survival rate of 66.88%; this, however, is not significantly different from day one.

Day Four

On the 4th day of culture there is 66.25% viability present which is significantly less than was present on day one. The total loss by day four was 33.75%.

Day Five

By the 5th day of culture there is 59.67% viability present or a loss of 40.33%.

Day Six

On this day there is a pronounced loss of viability to 48.02%.

This will be discussed at greater length later.

Day Seven

On this day the viability level is equivalent to the days 3 through 5, or by calculation, 62.77%.

Days Eight and Nine

During days 8 and 9, there was a 61.89 to 65.85% level of viability.

These dates are graphically portrayed in Figure 1. The data for day one through day nine is tabulated and analyzed by the "student T-test" in Table 4. Significance is evident only after day three and then at a low level, in most cases ($p = 0.05$). In other words the viability of these cultures from day 3 to day 9 is approximately 65%. The sharpest decline of viability occurs between the control and day one.

II. Survival of Anterior Pituitary Cell Types

A. Acidophils

Analysis of the intact fresh anterior pituitary gave 47.84% acidophils. These were counted using the PAS-Orange G procedure.

Day One

By day one, the number of acidophils had been reduced significantly to 36.53%, giving a 11.31% degranulation of acidophils in the culture medium.

Days Three, Four, and Five

By day three, the number of acidophils had stabilized to near the normal values where they remained through day 5.

Day Six

On this day, the acidophilic cell counts had risen sharply

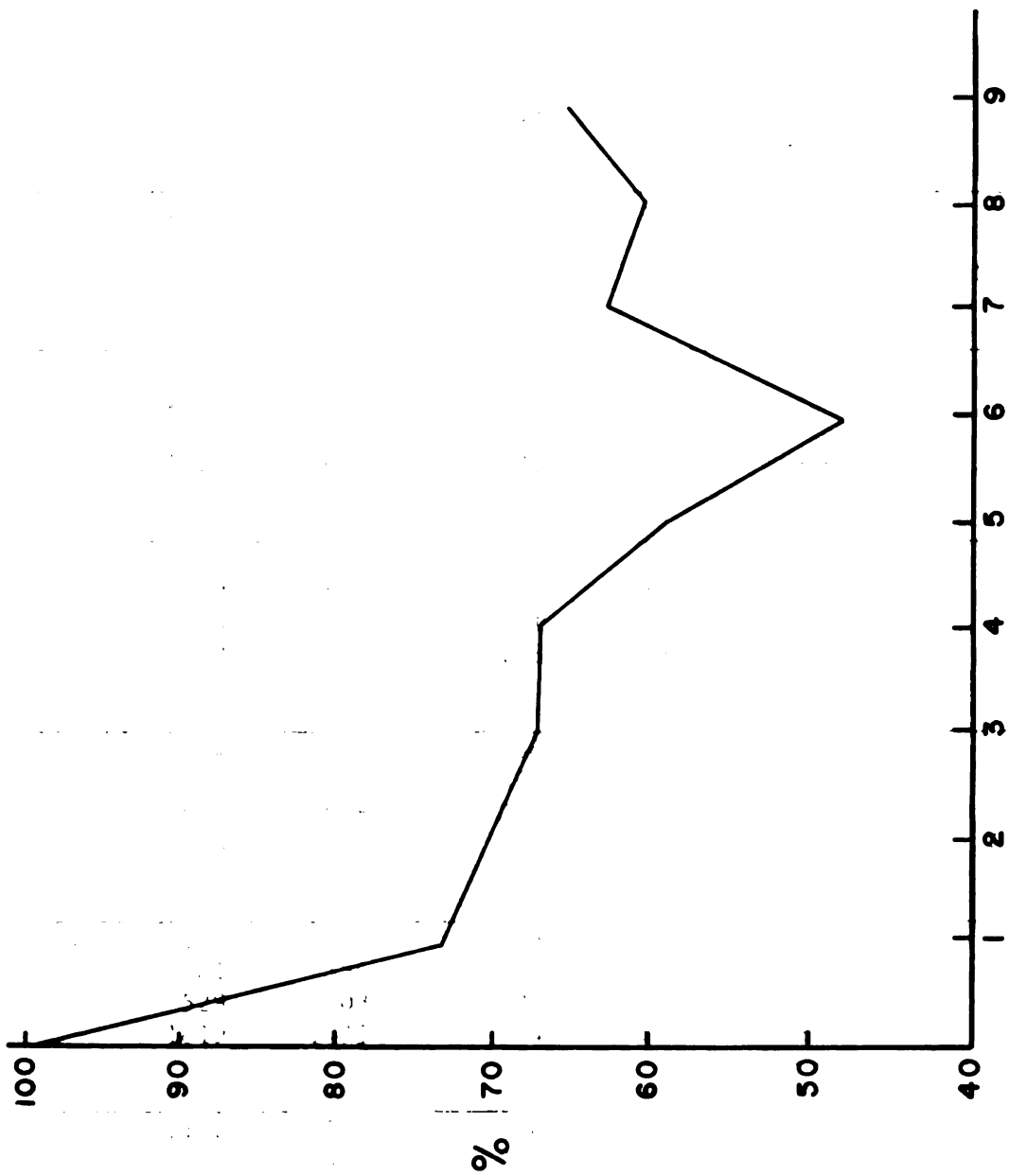

FIG. I. VIABILITY OF PITUITARY CELLS IN ORGAN CULTURE

FIG. 1. VARIATION IN CELLS IN ORGANO CULTURE TO VIABILITY

TABLE 4

VIABILITY OF ANTERIOR PITUITARY CELLS
IN ORGAN CULTURE 1-9 DAYS

Day	Total Cells	Total Viable	Total Non-viable	Percent Viable Cells	Average Percent	p
1	1174	750	424	63.80		
	1345	967	378	71.89		
	978	748	230	76.48		
	954	751	203	78.72	72.72 ± 4.88	
3	1156	713	443	61.67		
	857	581	276	67.79		
	1152	820	332	71.18	66.88 ± 3.47	NS
4	1323	750	573	56.68		
	1151	873	278	75.84		
	1100	798	302	72.54		
	1029	628	401	61.03	66.25 ± 7.66	.05
5	598	270	328	45.39		
	1470	899	571	61.15		
	860	399	461	46.39		
	760	652	108	85.78	59.67 ±13.78	.05
6	1314	576	738	43.83		
	907	309	598	34.06		
	829	548	281	66.10		
	946	455	491	48.09	48.02 ± 9.08	.01
7	1231	663	568	53.85		
	601	354	247	58.90		
	529	303	226	57.27		
	1819	1475	344	81.08	62.77 ± 9.33	.05
8	1192	722	470	60.57		
	658	416	242	63.22	61.89 ± 1.32	.01
9	1374	1077	297	78.38		
	1042	672	370	64.49		
	984	536	451	54.30		
	1079	715	364	66.26	65.85 ± 6.46	NS

p = level of significance compared to day one.

to 59.14% or a 11.30% increase over the normal range. This is the only day that shows any statistically significant differences from the normal values.

Days Seven, Eight, and Nine

On the following three days the number of acidophils were in the normal range, with perhaps a slight increase on day 9 to 51.50%.

B. Basophils

These were counted using the PAS-Orange G method. No attempt was made to differentiate between the types of basophils as the PAS will react with all basophilic cells. The normal intact pituitary showed a 10.50% level of basophils.

The relative numbers of basophils decreased at a significant rate over the 9 day period. By the 9th day the calculated percentage was down to 3.65%. This constitutes a loss of 65.23% of the total basophilic population.

C. Chromophobes

The normal level of chromophobes in this group of rats was 41.62%. Over the 9 day period of culture the percentage of chromophobes showed a response which was indirectly proportional to the response shown by the acidophils. In other words, as the acidophils decreased the chromophobes increased. Figure 2 and Table 5.

III. Comparison of Orange G Acidophils and Acid Fuchsin Acidophils

During the course of this investigation it became apparent that there was little correlation between the cell counts from the two methods of staining the cultured pituitary glands. When the values obtained at 3, 6, and 9 days of culture were plotted for each stain,

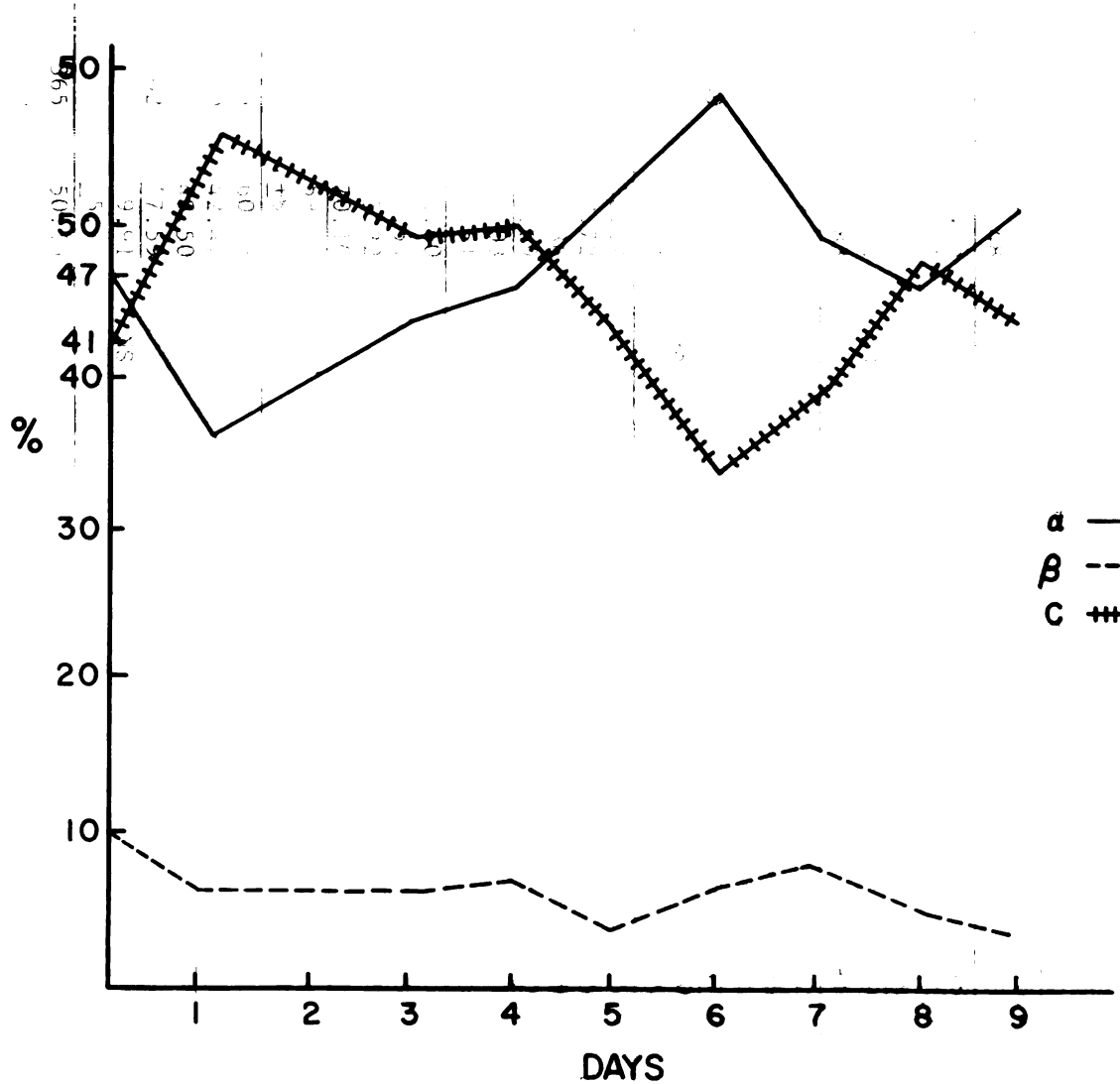

FIG. 2. CELL COUNTS OF CULTURED PITUITARIES BY PAS ORANGE TECHNIC

FIG. 5. CELL COUNTS OF CULTURES BY PAS CHANGE TECHNIC

TABLE 5

SURVIVAL OF ANTERIOR PITUITARY CELL TYPES
IN ORGAN CULTURE 1-9 DAYS

Day	Total A	% A	p	Total B	% B	p	Total C	% C	p
N	748	49.04		169	11.08		608	39.86	
	796	46.46		175	10.21		742	43.31	
	772	47.89		160	9.92		680	42.18	
	756	48.00		171	10.80		648	41.14	
		47.84			10.50			41.62	
		±0.69			±0.43			±1.12	
1	222	29.60		52	6.93		476	63.46	
	366	37.84		61	6.30		540	55.84	
	289	38.69		39	5.21		420	56.14	
	301	40.07		29	7.45		394	52.46	
		36.53	.01		6.47	.01		56.97	.01
		±3.48			±0.71			±3.24	
3	322	45.13		43	6.03		348	48.80	
	272	46.81		55	9.46		254	43.71	
	323	39.39		42	5.12		455	55.48	
		44.05	NS		6.87	.01		49.33	.01
		±2.83			±1.72			±4.10	
4	374	49.86		57	7.60		319	42.54	
	385	49.80		49	6.33		439	56.79	
	305	38.22		62	7.76		431	54.01	
	289	46.01		42	6.68		297	47.29	
		45.97	NS		7.09	.01		50.15	.01
		±3.87			±0.58			±5.32	
5	136	50.37		10	3.70		124	45.92	
	474	52.72		20	2.20		405	45.05	
	189	47.36		12	3.00		198	49.62	
	412	49.69		47	7.65		193	32.65	
		50.03	NS		4.14	.01		43.31	NS
		±1.51			±1.75			±5.33	
6	303	52.60		46	7.98		227	39.40	
	150	48.54		17	5.50		142	45.95	
	358	65.32		34	6.20		156	28.46	
	319	70.10		29	6.37		107	23.51	
		59.14	.01		6.51	.01		34.33	.05
		±8.57			±0.73			±8.34	
7	329	60.18		49	7.39		285	42.98	
	150	42.47		35	9.89		169	47.74	
	150	49.50		33	10.89		120	39.60	
	702	47.59		63	4.27		710	48.13	
		49.91	NS		8.10	.05		44.61	.05
		±5.11			±2.28			±3.32	
8	365	50.55		27	3.73		330	45.70	
	176	42.06		26	6.25		215	51.68	
		46.30	NS		4.99	.01		48.69	.01
		±4.24			±1.26			±2.99	
9	560	46.42		48	4.45		469	43.54	
	385	57.29		23	3.42		264	39.28	
	292	54.47		16	2.98		228	42.53	
	342	47.83		27	3.77		346	48.39	
		51.50	.05		3.65	.01		43.43	NS
		±4.37			±0.43			±2.53	

N = Normal animal; A = acidophil; B = Basophil; C = Chromophobe; p = Level of

against time, this phenomenon is readily observed (see Figure 3). The Orange G acidophils, as previously mentioned, show no appreciable increase by 3 days but increased by 6 days and thereafter maintained a level somewhat near the control values throughout the 9th day. The acid fuchsin acidophils on the other hand, decreased in a linear fashion from day one to a low of 5% on day 6 and remained at this low level through day 9. In addition when the 3, 6, and 9 day cultures utilizing the trichrome stain is compared to identical days with the PAS-Orange G method, significant differences were obtained at all levels (Table 6).

Also, it might be mentioned that cell counts from normal pituitaries using both stains indicated no significant differences.

IV. Prolactin Assay

Prolactin release was demonstrated in each culture of one through 9 days. The amount of hormone released during each culture period is tabulated in Table 7.

An analysis of variance (Snedecor, 1946) failed to reveal any significant differences in the release of prolactin between days of culture.

As a method of demonstration of a trend in prolactin release, the actual crop readings, after correction for dilution and conversion to international units, were plotted. The crop readings were listed in order of increasing values for each day of culture. These were then plotted, first the lowest readings, then the next lowest, followed by the next highest and finally the highest readings for each group (Figure 4).

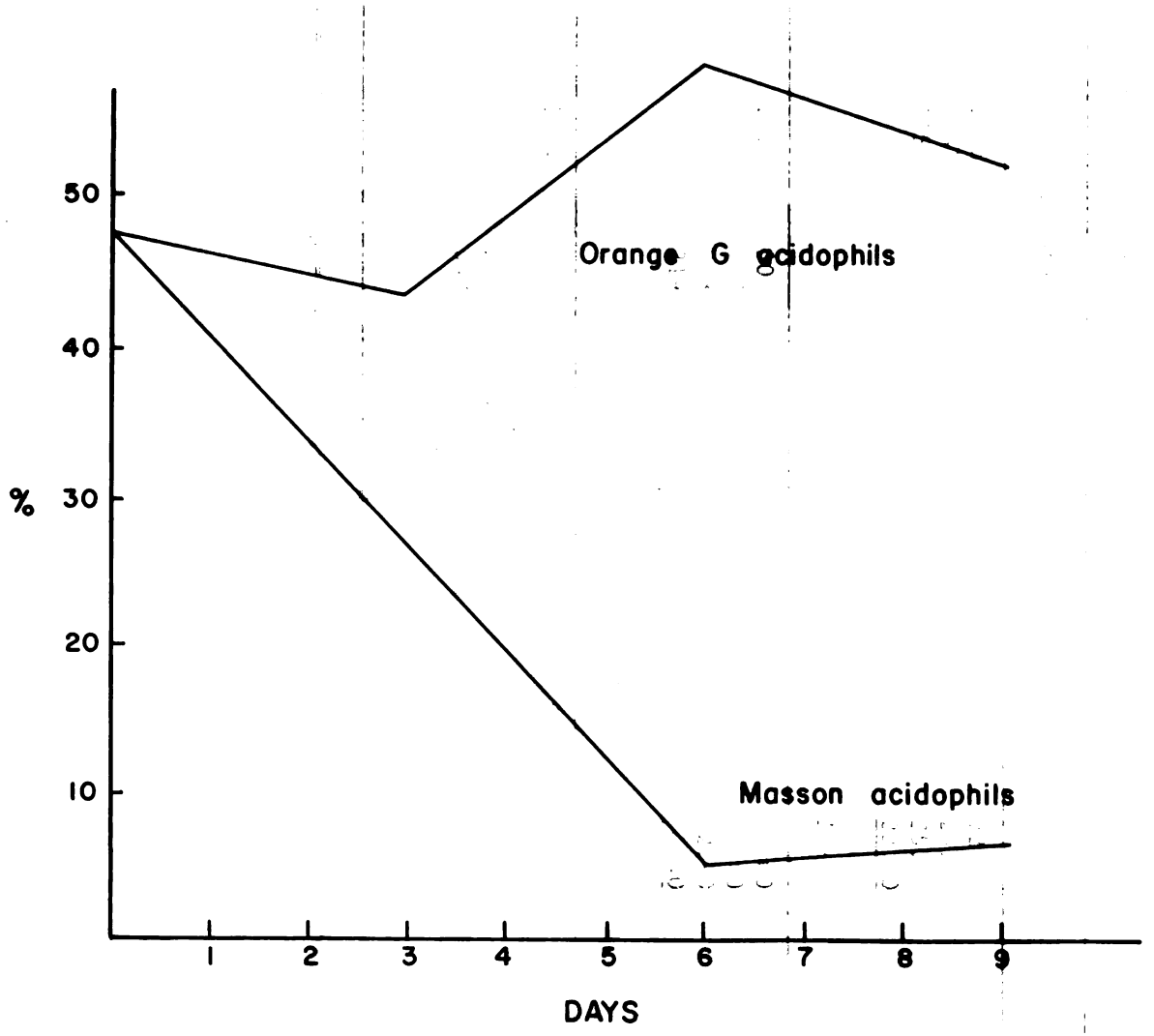

FIG. 3. COMPARISON OF ACIDOPHIL COUNTS BY TWO METHODS OF STAINING

FIG. 3. COMPARISON OF ACIDOPHILUS COUNTS BY STANDARD METHOD CMT

TABLE 6

ORANGE G ACIDOPHILS VS. ACID FUCHSIN ACIDOPHILS

Orange G Acidophils				Acid Fuchsin Acidophils			
Day	Total No.	Total A	% A	Total No.	Total A	% A	p
3	1156	322	45.13	1168	258	22.00	
	857	272	46.81	1042	248	23.80	
	1152	323	39.39	1075	278	25.80	
				1130	251	22.20	
			44.05			23.45	.01
			±2.83			±1.35	
6	1314	303	52.50	795	24	3.00	
	907	150	48.54	647	48	7.40	
	829	358	65.32	1043	31	2.90	
	760	319	70.10	872	39	4.40	
			59.14			4.42	.01
			±8.59			±1.48	
9	1374	560	46.42	896	82	9.10	
	1042	385	57.29	996	70	7.00	
	984	292	54.47	579	23	3.90	
	1079	342	47.83	939	67	7.10	
			51.50			6.77	.01
			±4.37			±1.44	

A = Acidophils, p = Level of significance compared to corresponding day.

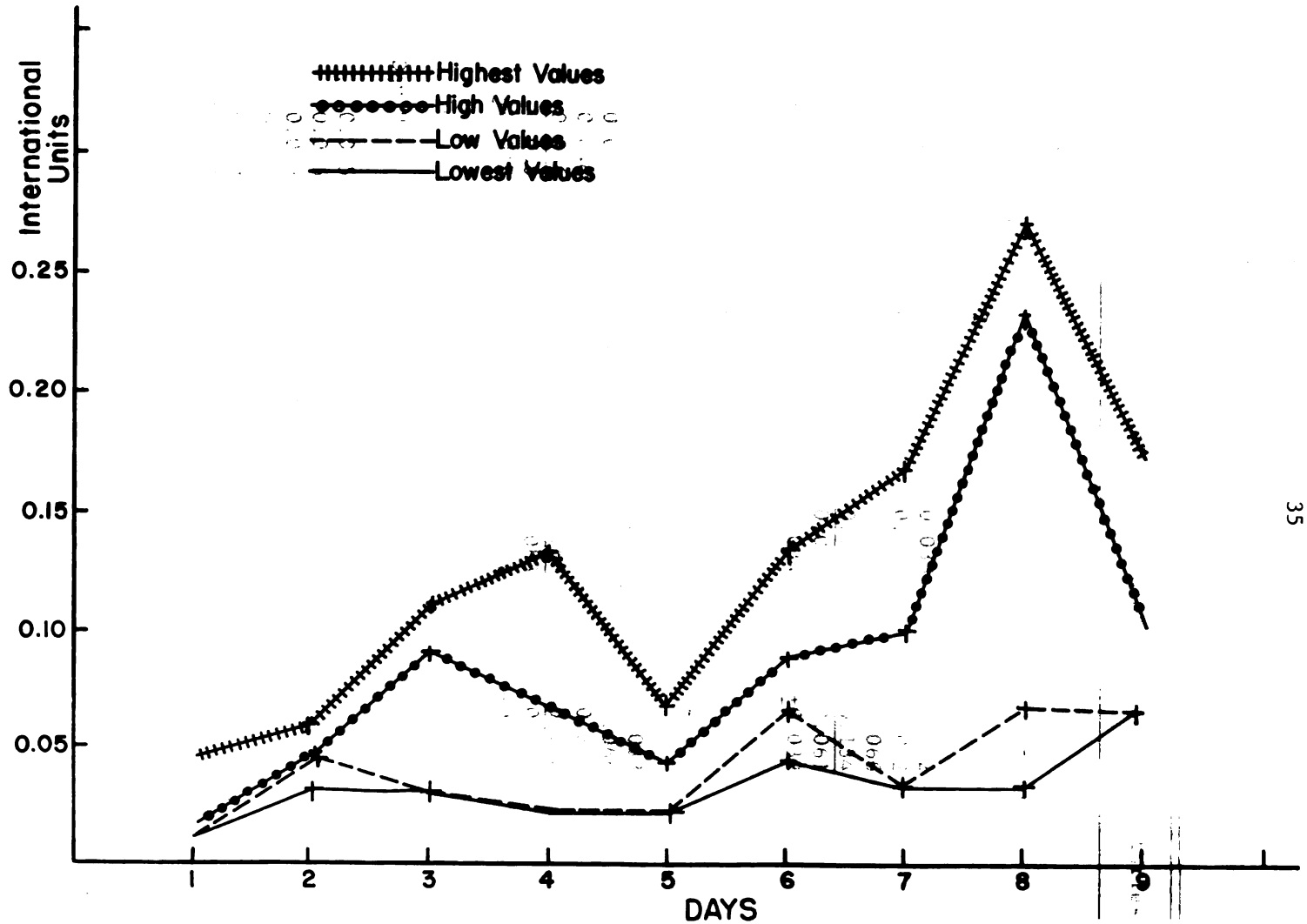

FIG. 4. PROLACTIN RELEASE INTO CULTURE MEDIUM

FIG. 4. PROLACTIN RELEASE INTO CULTURE MEDIUM
DAYS
1
2
3
4
5
6
7
8
9
0.00
0.10
0.15
0.20
0.25
Densitometric Units
Lowest Values
Low Values
Higher Values
Highest Values

TABLE 7

PIGEON CROP ASSAY OF CULTURE MEDIUM

Response is recorded in International Units Prolactin/bird/4 pituitaries

Day 1	2	3	4
0.011	0.033	0.033	0.022
0.011	0.045	0.033	0.022
0.011	0.045	0.090	0.066
0.045	0.060	0.112	0.134
0.019	0.045	0.067	0.061
±0.012	±0.006	±0.034	±0.039

Day 5	6	7	8
0.022	0.044	0.033	0.033
0.022	0.066	0.033	0.066
0.044	0.088	0.099	0.234
0.066	0.134	0.168	0.270
0.038	0.083	0.083	0.150
±0.015	±0.028	±0.052	±0.101

Day 9
0.055
0.066
0.099
0.168
0.099
±0.033

V. Microscopic Observations

Intact Control Pituitaries

Photomicrographs of the intact control pituitary cells are included to be used as a reference in evaluating the cultured pituitary cells. Most of the photomicrographs are self-explanatory and so no discussion is included other than the plate descriptions.

Cultured Pituitaries

Most of the areas of necrotic tissue in all the sections examined are confined to the center of the organ culture; however, in many instances there were necrotic areas along the edges. These areas on the edges probably resulted from poor contact with the oxygenated medium.

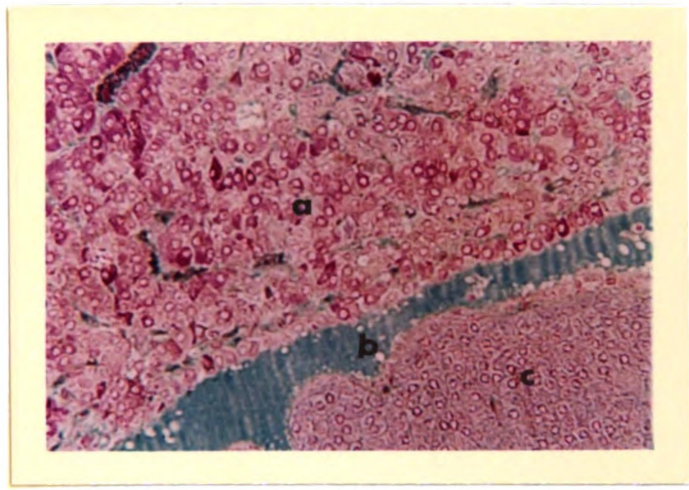

Fig. 5.--Intact Control Pituitary. Masson Trichrome and **Aldehyde Fuchsin stain.** 100X.
(a) Anterior lobe. (b) Hypophyseal cleft. (c) Inter-**mediate** lobe.

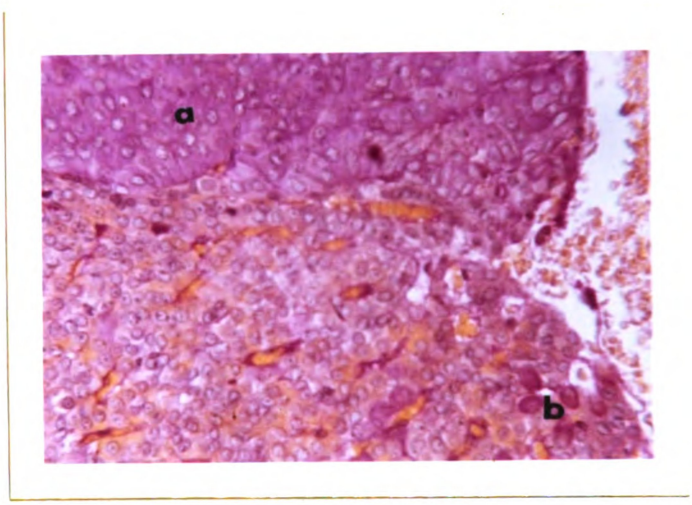

Fig. 6.--Intact Control Pituitary. PAS Orange Stain. 100X.
(a) Intermediate lobe. (b) PAS positive basophils.

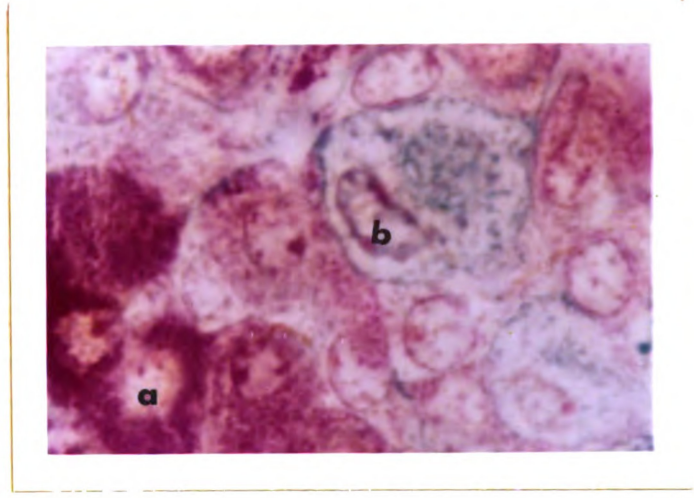

Fig. 7.--Intact Control Pituitary. Masson Trichrome and Aldehyde Fuchsin stain. 1250X.
(a) Large well granulated acidophil, probably LTH. (b) Large basophil presumably an LH secretor due to the kidney shaped nucleus.

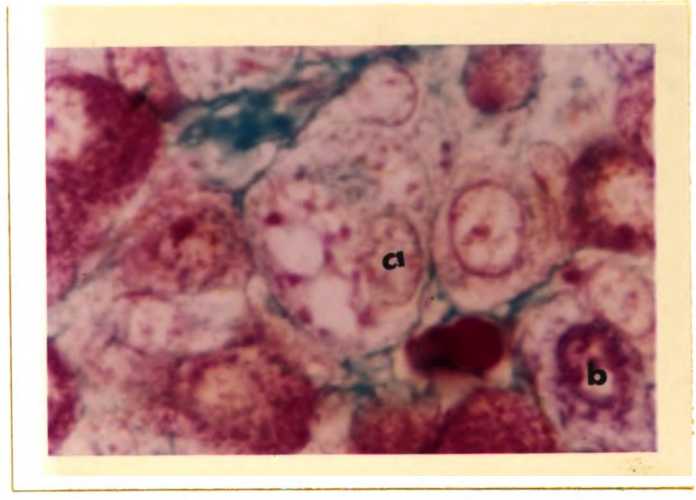

Fig. 8.--Intact Control Pituitary. Masson Trichrome and Aldehyde Fuchsin stain. 1250X.
(a) Large basophil with vacuoles filled with acidophilic material. (b) A TSH cell contains aldehyde fuchsin positive material throughout the cell but predominately around the nucleus.

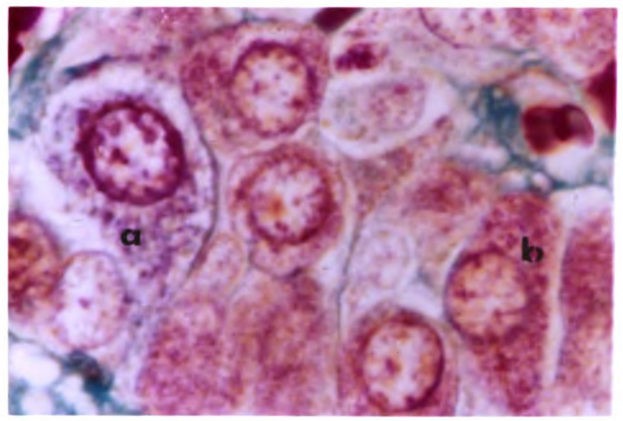

Fig. 9.--Intact Control Pituitary. Masson Trichrome and Aldehyde Fuchsin stain. 1250X.
(a) TSH cell, this is typical of the TSH cells found in the normal pituitary. (b) Acidophilic, columnar shape, probably STH cell.

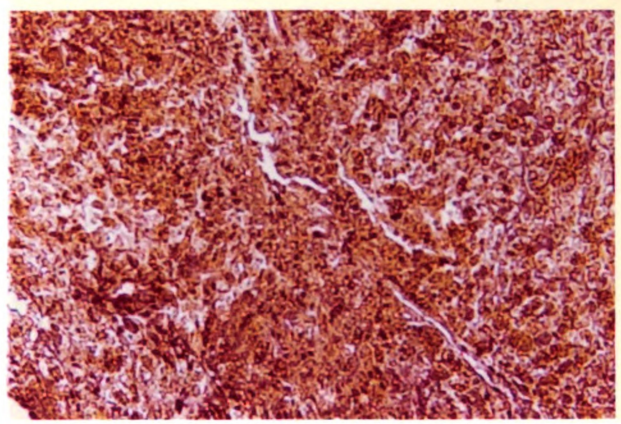

Fig. 10.--One-Day-Old Culture. PAS Orange G. 100X.
The viability appears good except in the central area.

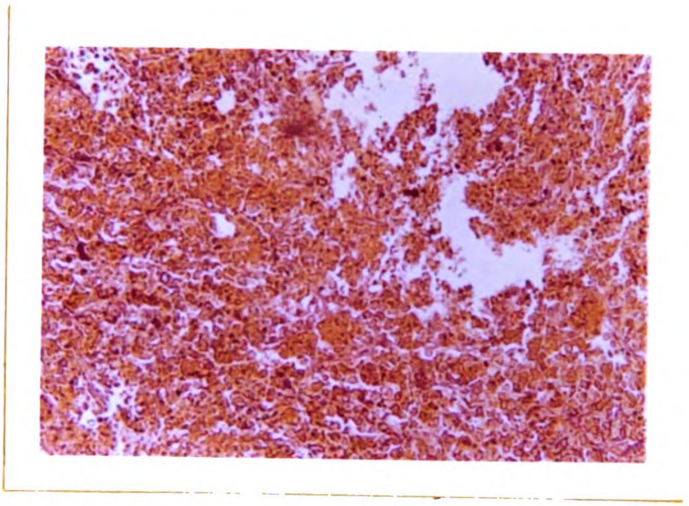

Fig. 11.--Three-Day-Old Culture. PAS Orange G. 100X
Viable tissue is present; however, the majority of this photograph is degenerating tissue.

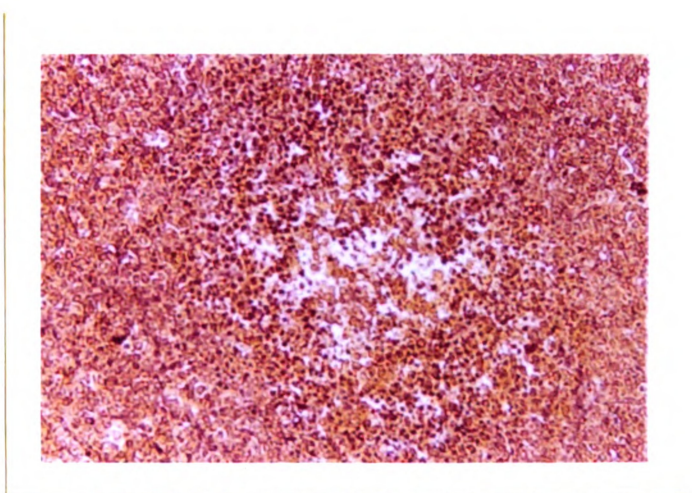

Fig. 12.--Nine-Day-Old Culture. PAS Orange G. 100X.
Viable tissue surrounds the central necrotic area.

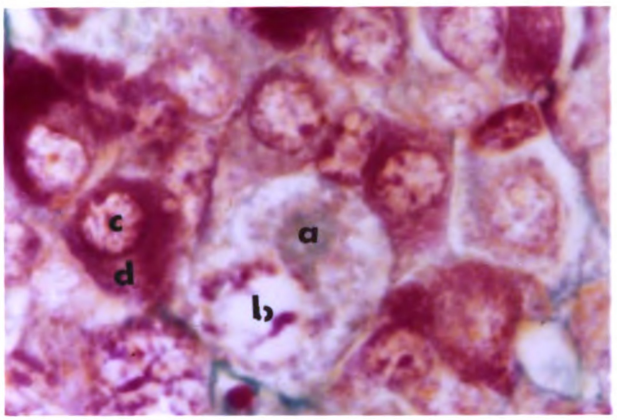

Fig. 13.--Three-Day-Old Culture. Masson Trichrome and Aldehyde Fuchsin Stain. 1250X.

In the center is a large basophil (a) containing a large vacuole (b). The acidophils (c) are dark and contain a pale area the negative Golgi image (d).

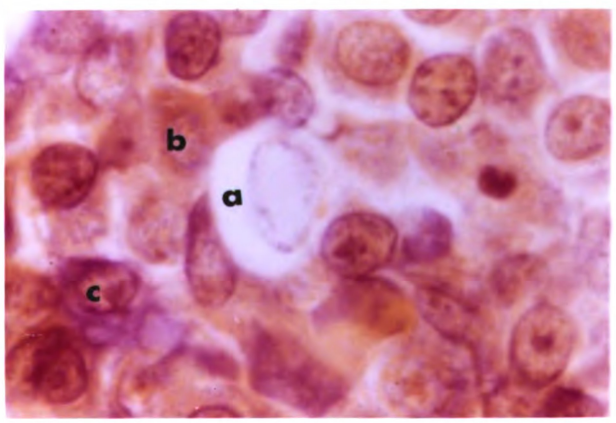

Fig. 14.--Four-Day-Old Culture. PAS Orange. 1250X.

A large hypertrophied chromophobe is located in the center, (a) the nucleus is very faint and hypertrophied. Around the nucleus is a slight rim of acidophilic material. The acidophils (b) appear active. The basophils (c) are somewhat below normal size in appearance.

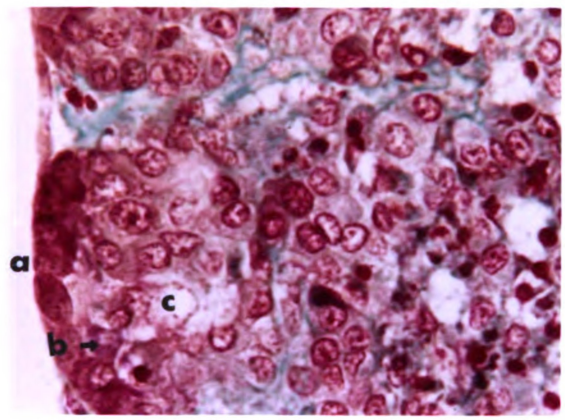

Fig. 15.--Nine-Day-Old Culture. Masson Trichrome and Aldehyde Fuchsin Stain. 400X.

Acidophils (a) are evident only along the edge. A small and degenerated TSH cell (b) is also present. A large basophil (c) displays a large vacuole.

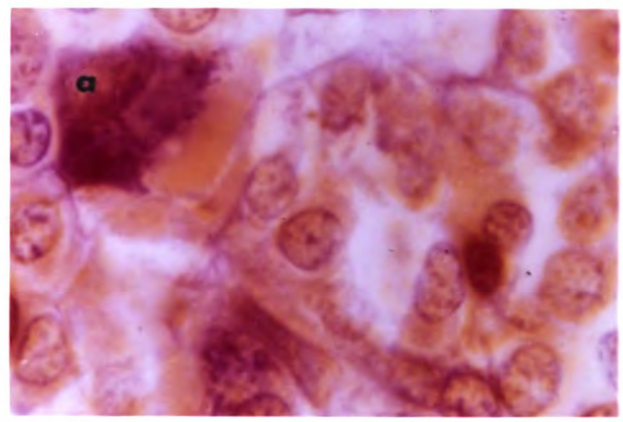

Fig. 16.--Nine-Day-Old Culture. PAS Orange G. 1250X.

(a) Prominent basophil with large PAS positive granules. Remainder of cells are weakly staining acidophils.

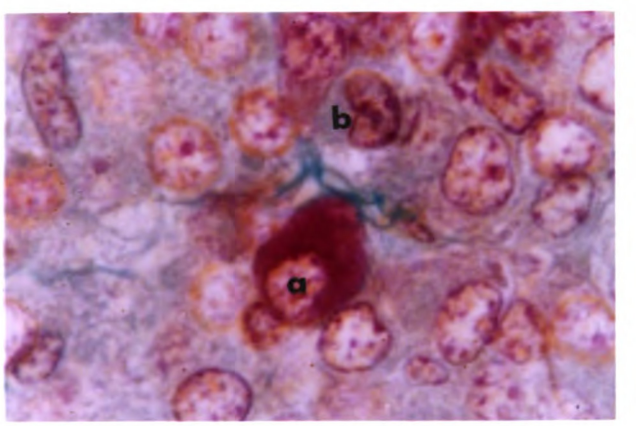

Fig. 17.--Nine-Day-Old Culture. Masson Trichrome and Aldehyde Fuchsin Stain. 1250X.

In the center is one of the very few acid fuchsin staining cells (a) present at nine days. Immediately above is a basophilic cell (b) with a kidney-shaped nucleus, presumably a LH cell. Notice the small cytoplasmic volume of both cells as compared to the normal cells in Fig. 7.

DISCUSSION

The results of this investigation demonstrate that the initial loss of viable cells occurs mainly during the first 24 hours. This is probably due in part to removing the anterior pituitary, slicing it into pieces, and the adjustment to an artificial environment. The losses after this period, that is between day one and day four, are possibly due to the normal degeneration of pituitary cells without the accompanying replacement of cells which occurs in the normal intact pituitary *in vivo*.

The amount of viable tissue after three days of culture stabilizes at the 60-65% viability level. Therefore when calculating prolactin production on the basis of pituitary weight the per cent of viable tissue should be considered. Also another consideration should be the per cent of acidophilic tissue present, since the basophilic and perhaps the chromophobic cells do not secrete prolactin.

From the results obtained here, the value of 47-48% acidophilic cells is acceptable for the majority of the one through nine days in culture. This value is also the figure determined for the intact control pituitaries. There are, however, some days when there are exceptions to this value, such as on day one, when the count is lower than the normal values. On this day, one may theorize that this is a reaction on the part of the prolactin producing cells to the absence of the hypothalamic control. Harris (1955) has shown that the hypothalamus has a direct inhibiting control over prolactin

secretion. This phenomenon has also been demonstrated in vitro by culture methods (Meites et al., 1961). It therefore seems logical that upon removal of this control the pituitary would react by an immediate release of prolactin. Now if one goes on the assumption that the prolactin is stored in the granules of the prolactin cells, then it is not surprising to see this disappearance of acidophilic staining cells.

Another day which merits some explanation is day 6. This particular culture showed the lowest viability of any culture, but probably this is the result of a poor technic in culture and not a reflection of 6 days in culture. However in evaluating the cytology, the number of acidophils exceeds the number found in the normal pituitary. One may postulate that at this stage the regranulation of the acidophils has increased to the point that all of the prolactin producing cells in the anterior pituitary have reached a stage of production where granules are present.

The assay for prolactin should verify these possibilities but due to the extreme variation in the pigeon crop assay and the limited number of observations, any variations between the days are lost. The stages of degranulation or regranulation could agree with the literature depending on the stage that the cultures were examined. Meites et al., (1961) reported that at 7 days the acidophils are predominant, while Schaberg and de Groat (1958) reported few acidophils at 12 days. At later stages the acidophils are in large numbers and heavily granulated for 21-28 days (Martinovitch, 1954).

The prolactin assay in this investigation does demonstrate one important point, namely that these cultures were active and capable of producing hormones. A second point is that these were male rats

and therefore were not expected to produce much prolactin.

The basophilic cell population decreases steadily during the culture period. This agrees with the conclusions reported in the literature. For example, the assay of the culture medium by Cutting and Lewis (1938) has shown that the basophilic hormone activity is present only during the first ten days of culture. Recently, Mittler and Meites (1964) have shown that the addition of hypothalamic extract to 3 day cultures can stimulate production of significant amounts of FSH. Ordinarily FSH is present in amounts which can be detected only for the first 3 days. After 9 days of culture, basophilic cells with large PAS positive granules were observed. (Figure 16). They were, however, somewhat smaller in size than the normal cells, but no evidence of degeneration was observed.

The chromophobic cell mass can conceivably include all of the cell types, the chromophilic cells in their degranulated stages (Severinghaus, 1937; Pearse, 1952), as well as the ACTH cells which contain granules but have no staining affinity (Siperstein, 1963), and also the chromophobic cells as such. Any massive degranulation by the acidophils would be shown by an increase in chromophobes and visa versa.

ACTH production has not been demonstrated after 4 days in culture without hypothalamic stimulation (Guillemin and Rosenberg, 1955). In this investigation ACTH cells were not identified in the normal pituitary; however, after 4 days in culture, an abnormal chromophobic cell appeared (Figure 14). A cell of this type has been reported by Rennels (1962). The origin of this cell could be from three sources: an extremely degranulated acidophil, an ACTH cell, or a large chromophobe. As far as the acidophil is concerned, most chromophilic cells

do not show an increase in size when they degranulate. Secondly these cells are only present in limited numbers, which would lead one to believe that this cell came from a group which was few in number to begin with, i.e. ACTH cells. However, as previously mentioned, ACTH production requires hypothalamic stimulation, so one would expect a degeneration of ACTH cell in culture. If this cell is merely a large chromophobe, it is difficult to see why it would become more prominent in culture, unless it were in some stage of hyperactivity. To sum up then, the significance of this hypertrophic chromophobe is not known.

When one analyzes the information obtained from the cell counts of Orange G and Trichrome stains, it is apparent that the acidophils are not of the same population. These curves may be compared to the information obtained for prolactin and growth hormone assays on cultured pituitaries. Nicoll (1962) reported that prolactin is produced at a relatively high level during the early stages of culture, mainly one through seven days. This corresponds to the increase of acidophils (orangeophils) observed during the first 6 days in this investigation. This increase is observed only when using the Orange G stain for acidophils, therefore these cells must be prolactin producers primarily. The curve obtained using acid fuchsin as an acidophilic stain indicates a pronounced decrease in acidophils from the beginning of the culture but particularly from day three to day six. This corresponds rather well with the results of the STH assay form cultured pituitaries. Deuben and Meites (1964) indicated that after the first 6 days of culture only very little STH is recovered, 19% as much as was recovered in the fresh pituitary. By calculation this investigation shows that after 6 days in culture, using Masson Trichrome

stain, there is only 9.4% of the original acidophils present. It therefore appears that these acid-fuchsin-staining cells are the producers of STH.

As I have indicated the LTH cell and the STH cell can be readily differentiated in some species; however, the rat has not proven to be one of these in most cases. Some results have been reported using modifications of Heidenhain's azan stain; however, these results have been sporadic and not reliable (Dawson, 1946). Lacour (1950) using pregnant or lactating rats has shown that the LTH cells are stained with Orange G, while the STH cells stained with erythrosin or axocarmine. Rennels (1962) has reported similar results but again his procedure has not proven to be repeatable. Pasteels (1963a) has reported very good results in differentiating the prolactin and STH cells using a tetrachrome stain (acid alizarin blue) developed by Herlant (1960). In this investigation the two types of acidophils in culture could not be differentiated on the basis of cytology. The only differentiation was through the use of the two stains mentioned.

There are several considerations which may be responsible for the variation in staining ability of the rat acidophil: (1) It is fairly well established that the acidophilic granules are a mixture of 4-8 proteins, (Landing, 1957) peptides, phospholipids, and hormones (Purves, 1961).

(2) The staining reaction is due to the acidophilia and not to the hormone components. The hormones LTH and STH can be dissolved out without loss of staining characteristics (Purves, 1961). Acidophilia may be due to the amount of arginine and histidine present in the cell (Herlant, 1964). The histidine content does not vary much in the normal LTH and STH cells (Glenner and Bagdaymon, 1960). Recent

studies indicate that there is a direct relationship between the abundance of secretory granules and prolactin content (Pasteels, 1963b). However in stages of hypersecretion of one or the other cell type they may vary considerably.

(3) Purves (1961) indicated that the granules vary in quality and quantity due to an alteration in the components which are affected by different rates of secretion. A well known example of this is seen in the LH producing cells of the dog or cat. When stained with Masson's Trichrome, these cells may appear red due to an excess acidophilic component, or may be green due to a change of acidophilic component, or may even be blue due to a staining by both acid fuchsin and light green. This is explained on the basis that the acidophilic and basophilic components vary during different rates of secretion.

(4) Pearse (1956) in a study of esterases in the hypophysis indicated that these enzymes increase significantly during pregnancy, and the RNA decreases in amount which may influence the staining characteristics. All of this information points to the fact that the staining affinity of the acidophils can vary during secretion in the anterior pituitary.

The outstanding point in this investigation is that by using two different histologic procedures, involving different fixatives and different acid stains, the acidophils can be shown to vary directly with the rate of hormones released.

There are several variables which can be considered in determining the difference in staining characteristics. Among these are the alterations in the biochemical make-up of the cell due to: secretion, culture procedures, different methods of fixation, different sites of attachment of the acid dyes, and the oxidation produced by the periodic

acid in the Schiff reaction.

Further investigation is needed in all of these aspects before any definite conclusion can be drawn on the exact components stained in this investigation.

SUMMARY

This study is a semiquantitation of the microscopic changes occurring in the pars distalis of male rats during periods of short term organ culture.

The parameters examined were: 1.) the amount of tissue surviving from 1-9 days of culture; 2.) the degree of survival of the major cell types; 3.) the correlation of the numbers of surviving acidophils, as identified by orange G and Masson trichrome staining, with prolactin and STH production.

The initial loss of viable tissue occurs in the first 24 hours of culture. Thereafter the viable tissue stabilizes at 60-65% of the normal amount.

The percentage of prolactin acidophils as detected by orange G staining increases from a normal value of 48% to 59% by the sixth day. By the ninth day the values returned to normal. The somatotropic acidophils demonstrated by Masson's trichrome stain decrease to 5% by the sixth day where they remain. The changes in the numbers of the two types of acidophils correlate with an increase of prolactin and a decrease of STH production. The numbers of viable basophils decrease slightly over the ninth day period.

In the normal rat, the two acidophilic cells can not easily be differentiated as to type, but in vitro during periods of hypersecretion of the prolactin cell and hyposecretion of the STH cell these two cells can be differentiated.

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APPENDIX

Histological Procedures

Four glands were removed on each successive day and ½ of each gland was placed in Bouin's fluid and the other ½ in formol-sublimate.

A. Masson's trichrome and Aldehyde Fuchsin (Baker, 1962). This procedure best demonstrates the acidophilic cells and the thyrotropic cells.

1. The gland is fixed for 24 hours in Bouin's fluid, prepared as follows:

   Bouin's fluid: 750 ml. of saturated picric acid in water.
   250 ml. of 37-40% formaldehyde.
   Add 1 ml. of glacial acetic acid to 20 ml. of the above solution, prior to use.

   Use 40 times as much fixative as tissue volume.

2. Dehydration

   a. 50% ethanol for 1 hour.
   b. 70% ethanol for 1 hour or may be stored in this solution.
   c. 80% ethanol, 2 hours or may be stored in this solution.
   d. 95% ethanol, 2 changes of 2 hours each.
   e. 100% ethanol, 2 changes of 2 hours each, render absolute with anhydrous MgSO4.

3. Clearing

   a. 100% ethanol plus carbon disulfide (CS2) in (1:1) overnight.
   b. CS2, 2 changes during 24 hour period.

(1) First change of CS2 must not be cloudy.

(2) The tissue at first will float and ideally eventually sink. Slight warming helps.

4. Infiltration with Paraffin

a. CS2 plus paraffin (1:1), use 56-58^{o} Tissuemat.

(1) Be sure Paraffin is cold when the CS2 is added.

(2) CS2 IS EXPLOSIVE, ABOVE 46^{o}C.

(3) Keep in a warm place, such as on top of a 60^{o}C oven. You must watch the stoppers on the vials containing the mixture so that they don't blow off and the CS2 evaporate. Allow 1-3 days for infiltration.

b. Place into the Paraffin at 56-58^{o}C and draw a vacuum, for 15-20 minutes, or until the bubbling stops. Requires 25 pounds of pressure. Without a vacuum oven, four changes of Paraffin at 15 minutes per change.

(1) Sectioning is best after a vacuum, otherwise the tissue tends to harden too much due to the prolonged heat.

5. Casting the infiltrated tissue in the block

a. Molds

(1) Glass concave molds work very effectively. They are heated and are coated with a film of glycerine.

(2) Hot paraffin (56-58^{o}) is poured directly into the mold and the tissue transferred before the paraffin cools enough to form a film over its surface.

(3) Metal molds may be used, it is not necessary to have glass molds.

b. Orientation of whole pituitaries in the mold

(1) The block is cast with the pituitary orientated so that the pars nervosa is directly flat on the bottom. Be sure that heated forceps are used to transfer the pituitary to the mold, as bubbles may form around the gland and this makes sectioning very difficult.

(2) The pituitaries taken from culture can not be orientated any particular way, because of the lack of identifying features.

6. Sectioning

a. Methods for obtaining representative samples from a whole pituitary gland.

(1) The pituitary is cut so as to take three representative levels through the gland. One level ¼ of the distance through the gland, a second ½ the distance through the gland, and a third level 3/4 the distance through the gland.

(a) For immature rats (22 days) begin by taking 12 sections at 6 microns to the ¼ level, then 30 sections at 4 microns. Then another 12 sections at 6 microns to the ½ level, then 30 more sections at 6 microns, and then 30 sections at 4 microns.

(b) For adult rats, begin by taking 30-35 sections at 6 microns to the ¼ level, then 30 sections at 4 microns. Then another 30-35 sections at 6 microns to the 3/4 level, and then 30 sections at 4 microns.

b. Required number of sections and thickness of sections from

each level to obtain a representative sample.

(1) Thirty sections cut at 3-4 microns, in ribbons of 10 sections are taken at each level.

c. Procedure for mounting sections:

(1) One ribbon of 10 sections from each of the three levels is mounted on a separate slide, so that three slides are obtained from a single gland, each containing a representative sample from each level.

(2) The 6 micron sections can be saved and mounted to be used as controls in the staining procedure.

7. Comments on sectioning and mounting of sections

a. In order to cut paraffin (Tissuemat) at 3-4 microns, the block must be kept cold at all times as must the knife.

(1) In order to do this, ice water is dripped on a piece of cheese cloth which is wound around the block.

(2) Also an ice cube is affixed to the surface of the knife blade to maintain a cold blade.

(3) The blade is wiped off periodically with chloroform to maintain a dry cutting surface.

b. The sections are affixed to the surface of a slide with albumen and allowed to dry one to several days at 37°C. The sections are not heated in any manner so as to melt the paraffin. Heating of the sections interfers with the selective staining of the trichrome stains. (Smith 1962).

8. Staining with Aldehyde Fuchsin and Masson's Trichrome

a. Xylol, 3 changes, 5 minutes each.

b. Absolute alcohol, 2 changes, 5 minutes each.

c. 95% ethanol, one change, 5 minutes.

d. 80% ethanol, one change, 5 minutes.

e. 70% ethanol, one change, 5 minutes.

f. Stain in Aldehyde fuchsin 20 minutes, or until the beta cells are stained.

   (1) Slides must be rinsed briefly in 70% alcohol and examined with the microscope.

   (2) A control slide should be processed at the same time.

g. Rinse in two changes of 70% ethanol, until the excess stain is removed from the background.

   (1) There should be no background staining if the stain is ripened properly. If the background does stain, there is little that can be done to remove it. The slide may be bleached, but this will change the selective staining ability of the aldehyde fuchsin stain. It is best to discard the slide and start again. The background should be absolutely clear.

h. Rinse in distilled water, 5 minutes.

i. Place in Masson A solution for 30 minutes, check with the microscope to determine the degree of staining of the alpha cells.

   (1) The slide may be rinsed in water briefly before examination.

j. Rinse in distilled water, as brief as possible.

k. Place in Masson B 30 minutes. This can be left as long as 1½ hours if necessary.

l. Place in Masson C for 45 minutes until the basophils are stained.

m. Rinse in distilled water to remove excess Masson C.

n. Place in Masson B for 2 minutes.

o. Place in 1% acetic acid, 2 minutes.

p. Rinse in distilled water.

q. Rinse in 95% ethanol, this must be brief, just long enough to remove the excess light green stain. About 10 seconds.

r. Place in absolute alcohol, 2-3 minutes, 2 changes.

(1) Too long will extract the light green.

s. Change rapidly to xylol for the absolute alcohol will pick up moisture from the air and the section will not clear in xylol. On microscopic examination the section will appear cloudy due to the moisture in the xylol.

(1) Change the slides through 5 changes of xylol of 5 minutes each.

t. Mount with Permount or Canada balsum.

9. Comments on staining

a. Acidophils will stain orange to red, but can't be distinguished as to type.

b. The thyrotrops or Beta cells of Halmi stain deep purple and are generally heavily granulated.

c. The gonadotropis or Delta cells of Halmi stain green with a flocculant cytoplasm.

d. Chromophobes may have a faint green cytoplasm, but the color is homogenous and distinct from the gonadotrop.

e. No nuclear stain is used as the acid fuchsin will generally stain the nuclei and nucleoli red; however, sometimes the light green will predominate in the nuclei.

10. Methods of Preparation of the stains

a. Aldehyde fuchsin (Elftman, 1959c).

(1) 500 mg. of basic fuchsin is dissolved in 100 ml. of 70% ethanol.

(2) Then add: 0.75 ml. of paraldehyde, and 1.25 ml. of concentrated hydrochloric acid.

(3) Allow the stain to ripen 26 hours in the oven at 37°C or 3 days at room temperature.

(a) The shade of the mixture will gradually turn to a deep purple, almost indistinguishable from gentian violet. As soon as this change has taken place the dye is ready for use.

(b) The stain retains its selective staining ability for about 3 days. Control slides should be processed before each group of experimental slides.

b. Masson A

(1) Acid fuchsin, 0.3 gm.

(2) Ponceau de xylidine, 0.7 gm.

(3) Distilled water, 100 ml.

(4) Glacial acetic acid, 1 ml. to be added just prior to use.

c. Masson B

(1) Phosphomolybdic acid, 1.0 gm.

(2) Distilled water, 100 ml.

d. Masson C

(1) Aniline Blue to saturation (about 2-3 grams), or Light Green to saturation, (about 2-3 grams).

(2) Distilled water, 100 ml.

(3) Glacial acetic acid, 2.0 ml. per 100 ml. of stain to be added just prior to use.

B. Periodic Acid Schiff and Orange G Method. (Purves and Greisbach, 1951). This procedure best demonstrates the basophilic cell types, but is perhaps the most useful in making cell counts.

1. Fixation
   a. The glands are fixed 24 hours in formol-sublimate which is prepared as follows:
      (1) Mix 9 parts of saturated mercuric chloride with 1 part of 40% formaldehyde.
   b. After 24 hours the glands are washed in 95% ethanol.
2. Dehydration
   a. Dehydration is continued from 95% ethanol and is the same as previously described under aldehyde fuchsin and Masson's trichrome stain.
3. The procedure for embedding and sectioning is the same as previously described.
4. Staining procedures (Elftman, 1959a)
   a. Xylol, 3 changes, 5 minutes each.
   b. Absolute ethanol, 2 changes, 5 minutes each.
   c. 95% ethanol, 1 change, 5 minutes.
   d. 80% ethanol, 1 change, 5 minutes.
   e. 70% ethanol, 1 change, 5 minutes.
   f. Lugol's iodine, 5 minutes.
   g. Rinse in distilled water.
   h. 5% sodium thiosulfate, 3 minutes.
   i. Wash in running tap water, 15-20 minutes.
   j. Distilled water, one change, 2 minutes.
   k. 1% periodic acid, 15 minutes.
   l. Rinse in 3 changes of distilled water, 2 minutes each.

m. Place in sensitized Schiff reagent (Elftman, 1959b), 20 minutes.

n. Rinse in tap water until there is a maximal development of color, 2-5 minutes. Rinse in clear water.

o. Stain in Harris hematoxylin, 2 minutes.

p. Rinse in tap water and follow by rinse in 1% HCl.

q. Wash in tap water, 10 minutes.

r. Stain in 3% Orange G at pH 2.0 for 10 seconds.

s. Rinse in tap water briefly, the intensity of the Orange G can be regulated during this and the next step.

t. Rinse in 95% ethanol briefly.

u. Absolute alcohol, 2 changes, 2 minutes each.

v. Xylol, 3 changes, 5 minutes each.

5. Comments on staining

a. Lugol's iodine is used to remove the mercury from the tissue sections. The mercury will show up on microscopic examination as dark crystals if not removed.

b. The sodium sulfate reduces the iodine to a colorless form which is more readily soluble and is removed by washing in tap water.

c. Schiff's reagent selectively stains all glycoprotein containing compounds in the pituitary. This includes 2 types of gonadotrophs; LH cells, and FSH cells as well as the TSH cells. Also the Schiff reagent will stain degenerative or necrotic cells.

d. The Orange G is a counterstain and as such stains everything that is not stained by the Schiff reagent. It does not, however, stain the chromophobes.

e. The Harris hematoxylin stains the nuclei of all cell types.

6. Preparation of stains

a. Schiff Reagent. (Elftman, 1959b).

(1) A stock solution is prepared by dissolving one gram of basic fuchsin in 100 ml. of distilled water.

(2) The solution is warmed to produce complete dissolution of the dye. The solution is then cooled and filtered.

(3) The next step is to produce sulfur dioxide by dropping sulfuric acid on sodium bisulfite in a closed container and then passing it through the basic fuchsin solution until decoloration occurs.

(4) The amount of dissolved sulfur dioxide determines the sensitivity of the Schiff reagent. The SO2 can be measured by titration against Lugol's iodine. The most effective Schiff reagent for staining rat pituitaries is produced when the titration requires 0.8-2.0 ml. of Schiff reagent to decolorize 1.0 ml. of Lugol's iodine.

(5) The amount of SO2 can be decreased by bubbling air through the decolorized Schiff reagent.

(6) The Schiff reagent must be stored in the refrigerator tightly stoppered to prevent deterioration. The solution remains effective over several months, but should be titrated periodically before use.

b. Orange G

(1) Three grams of Orange G is dissolved in 100 ml. of distilled water. Then the solution is adjusted to a pH of 2 with concentrated hydrochloric acid.

c. Harris Hematoxylin

(1) Dissolve 40 grams of ammonium alum in 400 ml. of distilled water by heating. At the same time dissolve 2 grams of hematoxylin in 20 ml. of absolute alcohol. Combine the two and bring to a boil. Add 1 gram of mercuric oxide slowly. After mixing, cool the solution in cold running water. Filter before use.