A CYTOPHOTOMETRIC ANALYSIS OF ANTERIOR PITUITARY CHANGES IN RATS EXPOSED TO REDUCED PRESSURE

RESEARCH REPORT

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FOREWORD

The present investigation was supported by Public Health Service Research Grant No. GM-05112 from the National Institute of General Medical Sciences, National Institutes of Health, Bethesda, Maryland. This report was prepared by Kenneth Rockwell and was submitted to the Graduate School in partial fulfillment of the requirements for the degree of Doctor of Philosophy at The Pennsylvania State University. The research was conducted by Dr. Kenneth Rockwell as a subproject of a research project entitled "Biophysical and Endocrine Changes in Acclimated Rats" with Dr. Adam Anthony, Professor of Zoology acting as the project director and Dr. G. K. Strother, Associate Professor of Biophysics as co-investigator. This work was also aided in part by funds from NASA Grant NGR-39-009-015(2).

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The experiments reported herein were conducted according to the principles outlined in the "Guide for Laboratory Animal Facilities and Care" and adopted by the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

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INTRODUCTION

The altitude-exposed rat is uniquely suited for an investigation of the pituitary during acclimation, since the endocrine state of hypoxia-exposed animals is well known from extensive studies of the thyroid, adrenal, and reproductive organs employing a multitude of biophysical and biochemical methods (34, 46, 54, 79). Investigations of the functional state of the rat anterior pituitary during altitude exposure are rare, however, and there are no available data which relate pituitary cytochemical changes to the process of acclimation.

Recent developments in the technique of visible cytophotometric analysis (62, 80, 81) permit, for the first time, a direct investigation of histochemically stained pituitary cell types and promise to further clarify the secretory response of individual cells during acclimation to environmental change.

A major aim of the present study is to describe the adaptive cytochemical responses of the hormone-producing cells of the rat anterior pituitary following exposure of varying duration to simulated high altitude. All data are obtained by application of differential cell count and analytical cytophotometric techniques. A related and supplemental aim is to cytophotometrically characterize the various pituitary cell types on the basis of their absorption spectra.

GENERAL CONSIDERATIONS

A. Statement and Definition of Terms.

In those animal forms in which aerobic cellular respiration constitutes the chief biochemical source of metabolic energy, the environmental oxygen tension is the major parameter to which the organism must respond, if it is to survive. Homeostatic mechanisms have been demonstrated which enable the organism to adjust to changes in such environmental factors (7), and there is general agreement that those mechanisms reach their highest expression in species which maintain a fairly constant internal environment in the presence of great changes in external conditions (64).

The French physiologist Bert was among the first to recognize the relationship which exists between altitude and the partial pressure of oxygen and was the pioneer in the systematic study of the effects of increased altitude upon the living organism. His observations of the external manifestations of altitude exposure demonstrated the physiological importance of adequate oxygen tensions and suggested the presence of mechanisms by which the individual could adjust to limited changes in altitude and the corresponding changes in barometric pressure (10).

In more recent years investigations in altitude physiology have made it clear that the response of the organism to hypoxic conditions is subdivisible into two parts (87). These may be designated as the initial or acute period of exposure which begins

immediately and persists for, at most, a few days and the prolonged or chronic period which may last for the life span of the individual. Each of these periods may be characterized by the physiological state of the organism, and each is sufficiently different so that Prosser (64) urges the use of special terminology to distinguish between them. Thus acclimation is defined as the "compensatory alterations seen in an animal exposed to controlled laboratory conditions," while acclimatization means "changes under natural conditions of climate, season, or geography." These definitions are retained throughout this report, and, in addition, the terms simulated high altitude, altitude-exposure, reduced pressure, and hypoxia are considered to be synonymous and are used interchangeably (54, 83).

B. Adaptation to Simulated High Altitude.

1. General mechanisms. The organismic changes which characterize full acclimation to conditions of reduced pressure are widely recognized and extensively reviewed elsewhere (78, 83). For summary purposes these may be grouped as: 1. functional adjustments which increase the exchange of oxygen between the environment and the organism such as hyperventilation and an increased residual air capacity of the lungs (39), 2. an hyperplasia and hypertrophy of certain components of the circulatory system, demonstrated as a marked polycythemia and an increase in the number and size of certain tissue capillaries (5, 56), and 3. metabolic and physicochemical reorganization at the tissue and

cellular level, which enhances cell survival in the presence of anoxemia (4, 70) and involves an increased dependency upon anaerobic sources of metabolic energy with a corresponding decrease in peripheral tissue oxygen consumption (6).

2. Neural mechanisms. The mechanisms which must operate at the onset and during the early phase of altitude exposure to bring about the acclimated state are not well understood. It is known that among the several body tissues the nervous tissue is especially sensitive to oxygen want (7, 31, 71, 83, 86, 90). Furthermore, the behavioral changes such as appetite loss, general lethargy, hyperventilation, and impairment of mental activity which accompany hypoxia exposure (34, 39) are controlled, in part, by neural reflexes, although the precise factors responsible for these effects are in no case fully elucidated.

3. Endocrine mechanisms. Considerable evidence shows the active participation of the endocrine system in the initial acclimation of the organism to simulated high altitude. The response of the adrenal, thyroid, and gonad are especially well studied. Thus, Gordon et al. (28) demonstrate an increase in rat adrenal weight following both continuous and discontinuous exposure at 250-280 mm Hg. Similar effects are observed in rats exposed to controlled gas mixtures with 15 and 10 percent oxygen content (46). Weihe (87) reports a maximum adrenal weight increase three days after the transport of rats to a mountaintop environment (3450 m) which corresponds closely with the time at which adrenal cortical

osmophilia is at a maximum (63). Several investigations show increased levels of adrenocortical steroids in the circulation during the first few days of hypoxia exposure (37, 39, 83, 87).

Equally profound adjustments occur in thyroid gland function under similar conditions (34, 54, 55, 79). In a recent study Nelson (54) reports a loss of thyroid gland weight and changes in uptake and turnover of I-131, in the intrathyroidal MIT/DIT ratios, in serum PBI levels, and in fecal and urinary clearance of labeled thyroxine during the first few days of continuous exposure of rats to 380 mm Hg. These data are interpreted as a transient functional hypothyroidism (55) which is of paramount importance in restoring the balance between available oxygen and oxygen utilization at the tissue level.

The observed responses of the gonad to high altitude conditions are less clear. Moore and Price (52), in an extensive study of acclimatization to altitudes slightly in excess of 14,000 feet, are unable to demonstrate any measurable changes in either the ovary and testis or in general reproductive function. Loss of testicular weight following both intermittent and continuous exposure to 25,000 feet of simulated altitude is reported by Gordon *et al*. (28) and confirmed under similar conditions by Altland (2), who also demonstrates a complete loss of reproductive function in all exposed animals. Assessment of the female response in both of the above studies leads to the conclusion that the ovary and female accessory reproductive structures are less affected than the

corresponding structures of the male. Additional investigations show that the immature testis is less susceptible than the adult organ to hypoxia (3). Further understanding of the effects upon the testis has been delayed by the observation that only the germinal epithelium responds to altitude exposure with relatively little change noted in the interstitial cells of Leydig (28, 83).

Adrenal, thyroidal, and gonadal responses to simulated high altitude are not possible without related changes in the function of the anterior pituitary, since all of these endocrine structures are known to engage in reciprocal interactions (82). An important aim of the present study is to determine the nature of these anterior pituitary adjustments in rats exposed to a reduced barometric pressure of 380 mm Hg.

C. The Histophysiology of the Anterior Pituitary.

Pituitary gland structure and function is the subject of several recent reviews (29, 36, 65 66), and it is clear that, despite more than a century of extensive study, our present concepts of this major endocrine organ are far from complete. The dual origin during embryonic development, the resultant heterogeneous cell population present in the adult structure, and the multiplicity of hormones secreted by the several parts of the gland make this structure unusually refractory to either descriptive or experimental analysis.

1. Development and structure. The pituitary is present in all vertebrate species. Structural variations between species are common,

but in a typical mammalian form like the rat the gland consists of several parts which reflect its embryonic development (65). Thus, the ectodermal cells of the embryonic structure known as Rathke's pouch become the glandular epithelia of the adult adenohypophysis, while neural ectoderm from the floor of the embryonic diencephalon contributes to the adult neurohypophysis. In final form the adenohypophysis includes the pars tuberalis, the pars distalis, and the pars intermedia, while the neurohypophysis consists of the pars nervosa and the infundibular stalk by which the entire gland maintains its attachment with the overlying hypothalamus. The cavity of Rathke's pouch commonly persists as the residual lumen within the adenohypophysis and permits an easy separation of the pars tuberalis and pars distalis from the remaining structures (58). These two are known collectively as the anterior lobe of the pituitary. In such forms the pars intermedia, pars nervosa, and infundibular stalk then form the posterior lobe of the gland. In the present investigation only the structure and function of the anterior lobe are of interest and further comments are confined chiefly to the pars distalis, since no endocrine function is known for the pars tuberalis (82).

2. Cell types in the pars distalis. It has been known for many years that the pars distalis is composed of more than one type of cell (61). While the initial distinction has been between the chromophobes, which do not bind appreciable quantities of dye following histological staining, and the chromophils, which stain with

relative ease, the continued application of a variety of histological techniques to the pituitary has led to an increasingly complex separation of chromophil cell types within the adenohypophysis (36, 66). The result is an ever more chaotic nomenclature for the specific cell types based upon either cell form and function taken together or morphological description alone. Recent attempts to resolve this dilemma and establish a universally acceptable system of names for individual cell types have met with only limited success (84), although the majority of investigators support the subdivision of chromophils into acidophils and basophils (61). In addition, considerable, irrefutable evidence has accumulated to indicate further possible subdivisions within each of these chromophil categories in certain appropriate species (65, 66).

3. Hormones. Following the now classic experiments of Smith (75, 76), which demonstrate the effects of hypophysectomy in the rat, it has become evident that the pars distalis secretes six physiologically and biochemically distinct hormones: somatotropic hormone (STH), lactotropic hormone (LTH), corticotropic hormone (ACTH), thyrotropic hormone (TSH), follicle stimulating hormone (FSH), and interstitial cell stimulating hormone (ICSH) (84). The first three of these are either proteins or polypeptides while the latter three are glycoprotein in nature (44). Among the several endocrine organs of the mammal, only the anterior lobe of the pituitary is known to secrete such a large variety of hormones with such a great range of physiological effects (82). Furthermore, although reciprocal interactions can be

demonstrated between the anterior pituitary and the adrenal cortex, the thyroid, and the gonad, it is increasingly unclear to what extent this involves direct action by the cells of the pars distalis, since secretion of the several anterior lobe hormones is known to be controlled by neural centers in the hypothalamus (25).

4. The "one hormone--one cell type" theory. In light of the complexities both in histological structure and in endocrine function displayed by the anterior pituitary, efforts to combine the morphological and physiological data into one conceptual framework have enjoyed only partial success. Chief among the ideas which have gained favor is the notion that each of the anterior lobe hormones represents the secretory product of a single morphologically distinct cell type (36, 65, 66, 67, 84). Support for such a model comes from several sources.

The use of histochemical techniques, particularly the periodic acid-Schiff (P.A.S.) reaction, to demonstrate the distribution of glycoproteins within the pars distalis (12) suggests that basophil cells are the source of TSH, FSH, and ICSH (67). The chemical reactions by which the staining of glycoprotein occurs are well known (8, 41) and it seems safe to assume that the P.A.S.-positive basophil granules do represent stored intracellular hormone (36). Less well understood, but no less valuable, is the aldehyde-fuchsin stain for thyrotropic basophils (17, 27, 33, 74). These techniques for staining basophils, coupled with an appropriate variety of counterstains for the demonstration of acidophils, permit the identification of as many

as five hormone-producing cell types in the pars distalis of favorable species such as the rat, monkey, dog, bat, and cat (65). The proposed names for these five cell types are: somatotropic cells, lactotropic cells, thyrotropic cells, FSH cells, and ICSH cells. The latter two types are combined frequently as gonadotropic cells (84).

Observations with the electron microscope confirm the secretory nature of the cells of the pars distalis and amplify the data obtained with the light microscope (36). Using alternate thick-thin sections Lever and Peterson (43) distinguish between two types of acidophil cell and between thyrotropic and gonadotropic basophils on the basis of the fine structure and distribution of secretory granules within the cells. Farquhar and Rinehart (23) present similar evidence for the presence of two types of gonadotrophs in the rat anterior pituitary, and ACTH-producing cell types are proposed by several investigators (22, 72, 73). This brings the total number of apparent hormone producing cell types to six in appropriate species.

In the absence of hormone-specific, histochemical techniques for electron microscopy, assay of the hormonal content of secretory granules from the several cell types of the pars distalis is based upon studies of isolated granules prepared by cell fractionation, differential centrifugation, and microfiltration. The results indicate that STH and LTH activity is associated with granules from acidophil cells, while TSH, FSH, and ICSH are found in basophil granules (40). Further study of the basophil granule fraction indicates that ACTH is found there also (60). The precise site of ACTH production, therefore, remains undetermined.

D. Effects of Altitude Exposure on Anterior Pituitary Function.

Studies of the changes in structure and function of the rat anterior pituitary gland during acclimation to simulated high altitude are rare, and the data are conflicting and fragmentary (83). A decrease in rat pituitary gland weight following continuous exposure (48 - 214 hours) to 25,000 feet of simulated altitude is reported by Gordon et al. (28), while discontinuous exposure to the same conditions for four hours per day produces no measurable weight change. Exposure to mountain environments at 14,000 feet for 60 days is reported to cause no change in rat pituitary weight (52) and similar results are obtained for animals exposed to artificial atmospheres low in oxygen (46). Histological studies of anterior pituitary tissue from animals subjected to a variety of hypoxic conditions show an increased number of basophil cells with attendant changes in stain intensity, degree of cell granulation, and cell size and appearance (28, 59, 63), and a reduced number of acidophil cells (28, 63). Bioassay determinations of intrapituitary hormone concentrations reveal an increase in adrenocorticotropic hormone (ACTH) which is related to the extent of oxygen deprivation but independent of exposure duration (46). Intrapituitary thyrotropin levels remain unchanged following discontinuous exposure to reduced pressure, but appear to fall significantly after 48 hours of continuous exposure, while the gonadotropin content of the gland is increased following all types of hypoxia exposure (28).

The considerable variation in duration, severity, and nature of hypoxia exposure makes comparison of results difficult, if not impossible. None of the reported histological studies utilize the accepted, histochemically specific stains for pituitary cell types; hence cellular identifiction is entirely without functional correlation. In addition, there is no reported attempt to combine cell count data from the pars distalis of normal and altitude-exposed rats with a direct quantitative determination of intracellular pituitary hormone levels from histochemically stained tissue sections to ascertain possible relationships between numbers of cells of each type present and amounts of stored intracellular hormone.

The present study demonstrates the feasibility of this latter approach and employs two rather dissimilar analytical techniques to characterize the pars distalis of the normal rat and rats exposed for varying periods to a simulated high altitude of 18,000 feet. First, histochemically stained sections are analyzed by the classic, but still valid, technique of cell counting to determine whether altitude exposure induces a change in relative numbers of acidophils, thyrotropic cells or gonadotropic cells. Second, cytoplasm from individual cells from the same histological sections used for cell counting are examined with a microspectrophotometer to obtain absorption spectra characteristic for each cell type and to secure quantitative estimates of relative intracellular hormone concentrations.

E. Microspectrophotometry.

Cytophotometric techniques have proved to be of great value in the localization, measurement, and analysis of various intracellular components. The early work of Caspersson (11) in which cellular nucleic acid distributions and concentrations were determined by measurement of natural absorption of ultraviolet light at 2570 Å has been repeated for a large variety of tissues (85). Extension of the technique into the visible range of the electromagnetic spectrum has permitted study of both naturally occurring pigmented cellular entities and additional cellular structures stained by either empirical or histochemical procedures (62, 80, 89).

The special histophysiological problems which occur in the study of the anterior pituitary seem particularly susceptible to investigation by microspectrophotometric methods. Van Oordt (84) points to the desirability of having a few, "well-standardized," techniques for staining the pars distalis to overcome some of the confusion in cell nomenclature. Cytophotometric studies during the staining process would aid such standardization. Preparation of absorption spectra characteristic of cell types in stained tissue sections facilitates identification of individual cells and permits comparison with similarly stained cells from other sources (19, 21, 69). Spectral analysis of cellular constituents stained after cell fractionation and differential centrifugation yields information on cellular sites where dye binding occurs and aids in determining the effects of dye mixtures on the staining of individual structures (9).

Comparison of spectra of purified pituitary hormones stained *in vitro* with the P.A.S. reaction with the absorption spectra obtained from similarly stained intact pituitary cells confirms the chromophil source of the hormones (21), and quantification of intracellular hormone contents under normal and experimental conditions appears possible (81).

Despite the value of microspectrophotometric analysis in furthering our understanding of the structure and function of the anterior pituitary gland, studies employing this method are rare. A major aim of the present investigation is to utilize cytophotometry to characterize the chromophil cells of the pars distalis of normal and altitude-exposed rats and to obtain relative quantitative measurements of intracellular hormone content under normal and experimental conditions.

F. Restatement of the Problem.

The present study seeks to determine the histophysiological changes in the anterior lobe of the rat pituitary gland during initial exposure and acclimation to a simulated high altitude of 18,000 feet. All data are obtained from either fixed whole pituitary glands or tissue sections histochemically stained with alcian blue, periodic acid-Schiff, and picric acid. A statement of specific aims follows:

1. To establish whether altitude hypoxia induces any alterations in the relative proportions of chromophobe and chromophil cell types within the pars distalis.

2. To determine the effects of acute and chronic hypoxia on the cytochemistry of functional pituitary cells as reflected in alterations of their spectral characteristics.

3. To assess the effects of simulated high altitude upon the secretory activity of individual hormone-producing cells of rat anterior pituitary by quantitative cytophotometric analysis.

MATERIALS AND METHODS

A. Experimental Animals, the Decompression Chamber, and Histological Technique.

All pituitary tissue came from 90 Sprague Dawley male rats (Sprague Dawley Co., Madison, Wisconsin) used in an investigation dealing with thyroidal, adrenal, and blood chemical aspects of altitude acclimation. A portion of this work was conducted in cooperation with the U. S. Army Medical Research Unit, Fort Knox, Kentucky. Data on these rats in separate studies conducted by other graduate students included: radiochromatographic analyses of thyroid gland hydrolysates (54), fluorescence chromatography of adrenal gland corticosterone (37), flame photometry of serum and urinary electrolytes (15), myeloid (38) and lymphoid (24) responses, and electrophoretic analyses of serum proteins (56). The availability of pituitary material from rats whose metabolic, hemal, thyroidal, and adrenal responses were well characterized was a fortunate circumstance which greatly facilitated the present investigation.

The decompression chamber employed is that used by Anthony and his co-workers (Strickland, Harclerode, Ziegler, Nelson, Frehn, Ferguson, Mallette, De Angelo, Strother, Ackerman, and Hunt) from 1959 to 1966 in the altitude physiology research laboratores at The Pennsylvania State University (4), Plate I. It is constructed of concrete and is equipped with a viewing window and interior lighting. The total interior volume is 286 cubic feet, and when operating at

one-half atmospheric pressure the rate of air turnover is eight cubic feet per minute. Access to the chamber during operation is possible through a decompressible lock which enables the care and maintenance of experimental animals under continuous exposure to simulated high altitude for extended periods of time. The chamber temperature was 26 ± 1 C, while that of the room housing control animals was 25 ± 1 C.

The rats were divided into five groups. Group I, consisting of 15 animals, served as controls and was kept at an ambient pressure of 735 mm Hg, which is ground level (1200 feet) at University Park, Pennsylvania. Groups II-V were exposed to a simulated altitude of 18,000 feet (380 mm Hg) for varying periods of time: 19 rats exposed for one day (Group II), 17 rats exposed for two days (Group III), 14 rats for seven days (Group IV), and the remaining 25 rats had 30 days of exposure (Group V). Altitude exposure constituted the only experimental stressor. Each group of experimental animals was placed in the decompression chamber on a specified date so that all animals would be killed by exsanguination and have pituitary glands removed on the same day (see Table 2, page 37). From each of the five groups of pituitary glands so obtained, five individual glands were selected for differential cell counting and cytophotometric analysis of the cells of the pars distalis.

Dissected pituitary glands were fixed in 4% formalin (10% stock formaldehyde). After fixation was complete, but before further treatment, each gland was weighed on a Roller Smith torsion balance (sensitivity = 0.2 mg). All glands were then embedded in paraffin

with the aid of the Auto-technicon using routine procedure. Selected paraffin sections of anterior pituitary were cut at six microns and utilized in a preliminary study which attempted to find that particular staining technique best suited to the further pursuit of the major problem. Several specific staining procedures for rat anterior pituitary cytodifferentiation were employed in these preliminary histological studies: 1. The Wilson and Ezrin stain (88) which used periodic acid-Schiff (P.A.S.), methyl blue, and orange G, 2. Gomori's aldehyde fuchsin stain (27) for anterior pituitary thyrotropic cells, counterstained with orange G, 3. Elftman's combination of aldehyde fuchsin and P.A.S. (18) for differentiating anterior pituitary basophil cells, 4. The aldehyde thionine-P.A.S. stain of Paget and Eccleston (57), and 5. Mowry's stain (45) employing alcian blue, P.A.S., and picric acid.

The rat pituitary glands from control animals, one day, exposed two day, seven day, and 30 day exposed animals (five glands from each group-total of 25) which had been selected for differential cell counts and cytophotometric analysis were sectioned serially at four microns. All glands were oriented so that sections were cut in the coronal plane beginning on the dorsal side. Selected sections from each of these 25 series were deparaffinized, hydrated, and stained with alcian blue-periodic acid-Schiff,[1] plus picric acid for contrast

[1]Alcian blue, C. I. No. 74240, Matheson, Coleman and Bell, No. B804. Basic fuchsin, C. I. No. 42510, Harleco (batch LF-22), Arthur H. Thomas Co.

and counterstaining (45). The specific staining procedure has been included in Appendix A. Stained slides were dehydrated, cleared, and mounted in Permount.

B. Cell Counting Procedure.

The counting technique employed in analyzing the cellular composition of the five control and 20 experimental rat pituitaries was a modification of the method first suggested by Rasmussen and Herrick (68). From each of the 25 series of pituitary gland serial sections five sections were selected for counting: one section from near the dorsal gland surface, a second section from one fourth the distance from dorsal to ventral sides, a third section representing the midpoint of the dorsal-ventral distance, a fourth section located three-fourths of the distance from the dorsal surface, and a fifth from close to the ventral side of the gland. Each of these five sections was surveyed by making three scans with the microscope (Fig. 1). Scan A began at the approximate center of the section and proceeded to either the right or left lateral extreme of the section. The direction was selected to include the greatest possible number of counted fields. Every other microscope field was counted. Scan B began at the anterior edge of the section, about one third of the distance from the lateral extreme, determined by Scan A, to the center, and proceeded to the posterior edge of the section with every other microscope field being counted. Scan C was identical to B, but began about two thirds of the distance from the same lateral extreme

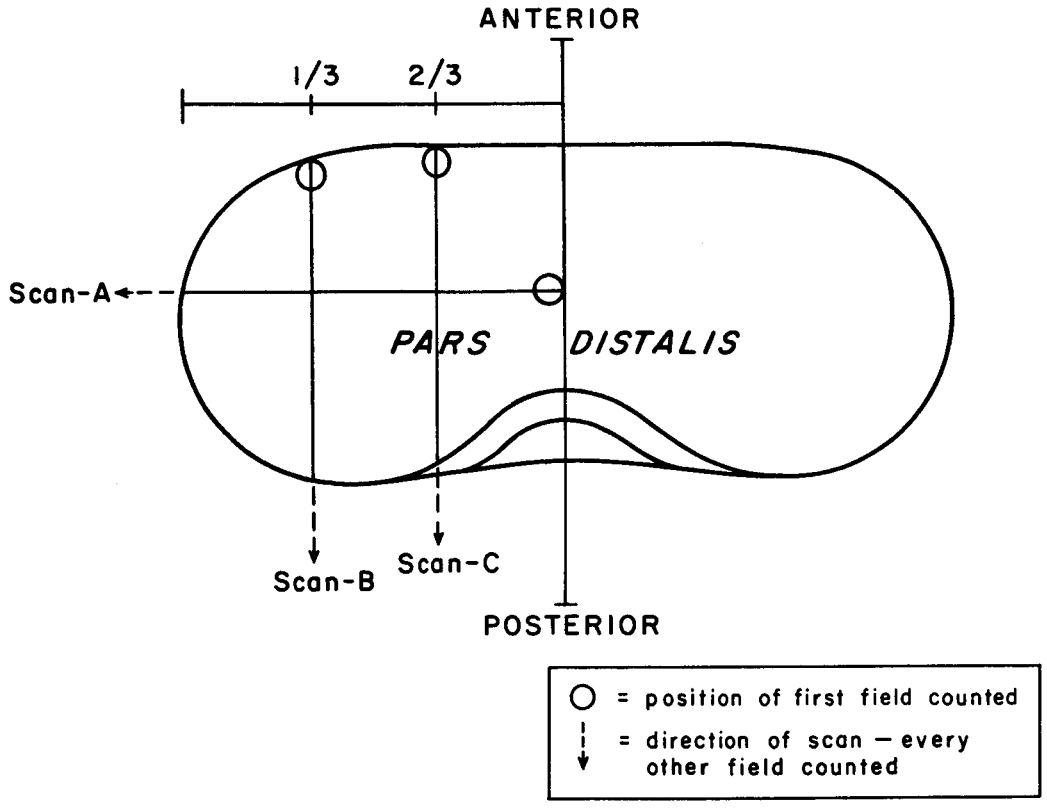


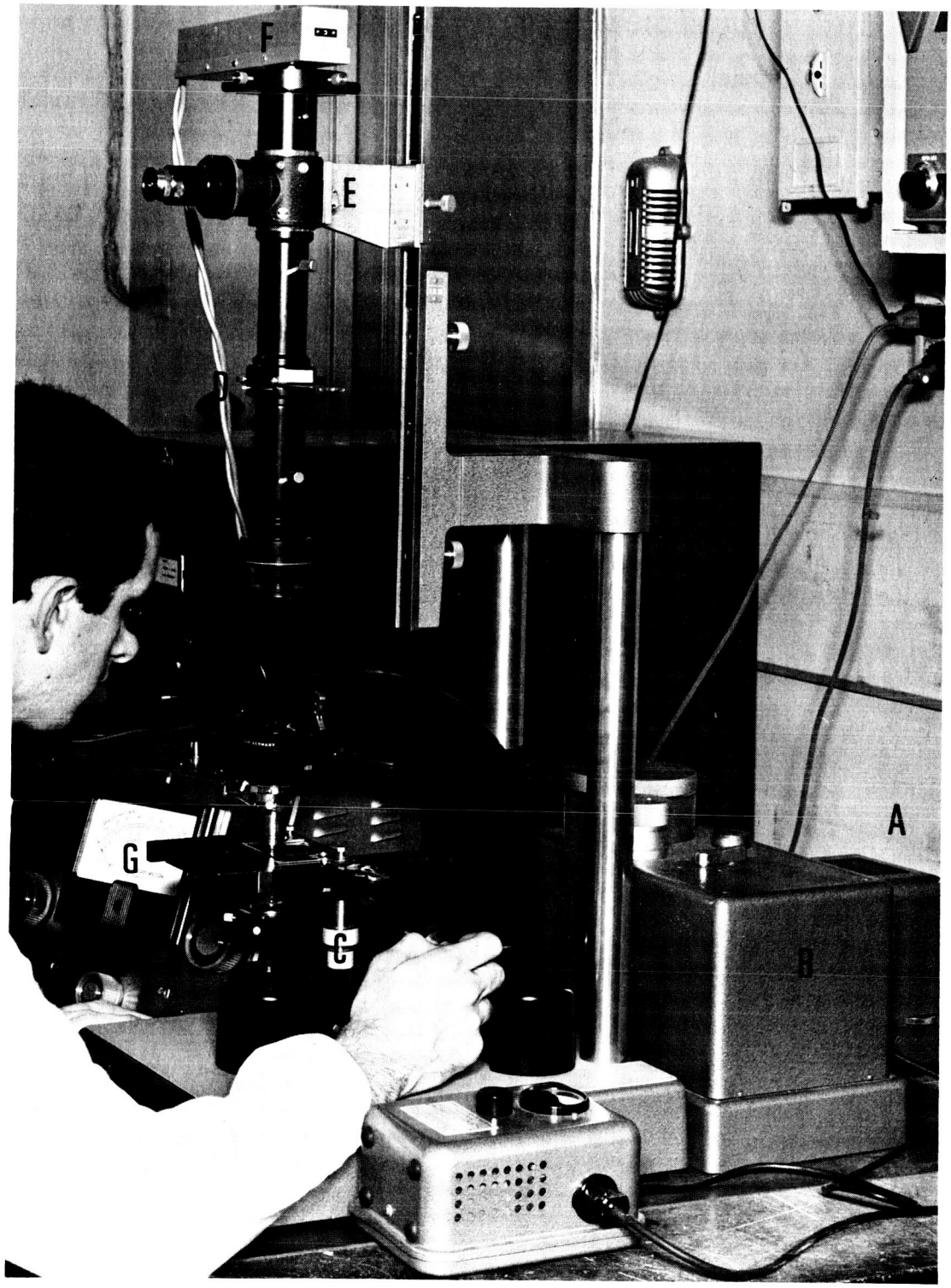
FIG. 1 PLACEMENT AND DIRECTION OF CELL COUNT SCANS SHOWN ON A MID-CORONAL RAT PITUITARY SECTION.

to the center of the section. In this manner cell count data were obtained from 15-20 fields per section and a total of 75-100 microscope fields per whole pituitary gland.

Individual microscope fields were counted using a cross-hair inserted in the ocular, and totals were obtained for all identifiable cells present. Portions of cells were included in the count if their identity was clear. All count data were obtained with a Bausch and Lomb, Dynazoom, binocular microscope using a 97 x /1.30 oil-immersion, achromatic objective, 10 x wide-field oculars, and "zoom control" set at 1.5 for a total magnification of 1455x.

C. Microspectrophotometric Analysis.

Microspectrophotometric data were obtained with a Leitz cytophotometer (E. Leitz, Inc., New York, N. Y.). This instrument, Plate II, consisted of the following major components: 1. a Leitz lamp housing with prefocused six volt, tungsten filament lamp and regulating, constant-voltage transformer, 2. a Leitz (Model IKAMS) dual prism, linear mirror monochromator, 3. a Leitz Ortholux microscope equipped with fluorite oil-immersion objective (FL oil, 95 x /1.32) and an achromatic objective (42 x /0.40) mounted as the substage condenser, 4. a Leitz optical train and mirror reflex housing, including a series of fixed photocell apertures of different diameters which could be positioned over selected areas of the specimen image to block off all light except that coming from the area of the specimen being analyzed, and 5. a Photovolt photomultiplier and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power unit (model 520 m). The relative relationships of these units to each other is illustrated in Figure 2.

After careful centering of the light source and adjustment of the optical train, an initial evaluation of instrument performance was carried out by obtaining a transmission spectrum for a Corning CS1-60 didymium glass filter and comparing the results with the spectral data provided by the manufacturer. The effect of monochromator exit slit width on spectral resolution was determined by comparison of absorption spectra for the same filter (CS1-60) obtained with exit slit widths of 0.8 mm, 1.6 mm, and 0.3 mm. Additional checks of instrument response involved a determination of the linearity of photocell response for the several available photocell apertures at a standard wavelength of 550 mμ using the 100x scale of the voltmeter.

From each of the 25 pituitary glands absorption spectra were obtained for all chromophil cell types demonstrated by the staining technique. Acidophil cells, thyrotropic basophils, and gonadotropic basophil cells (three of each type) were picked at random from the pars distalis of each gland and examined with the microspectrophotometer. Individual cells of each type always were selected from different histological sections. Cells were positioned prior to cytophotometric analysis with respect to the appropriate photocell aperture using the viewing ocular of the microspectrophotometer. All spectra in this study were recorded from a 4.0 micron diameter area of cell cytoplasm. The mechanical entrance and exit slits of

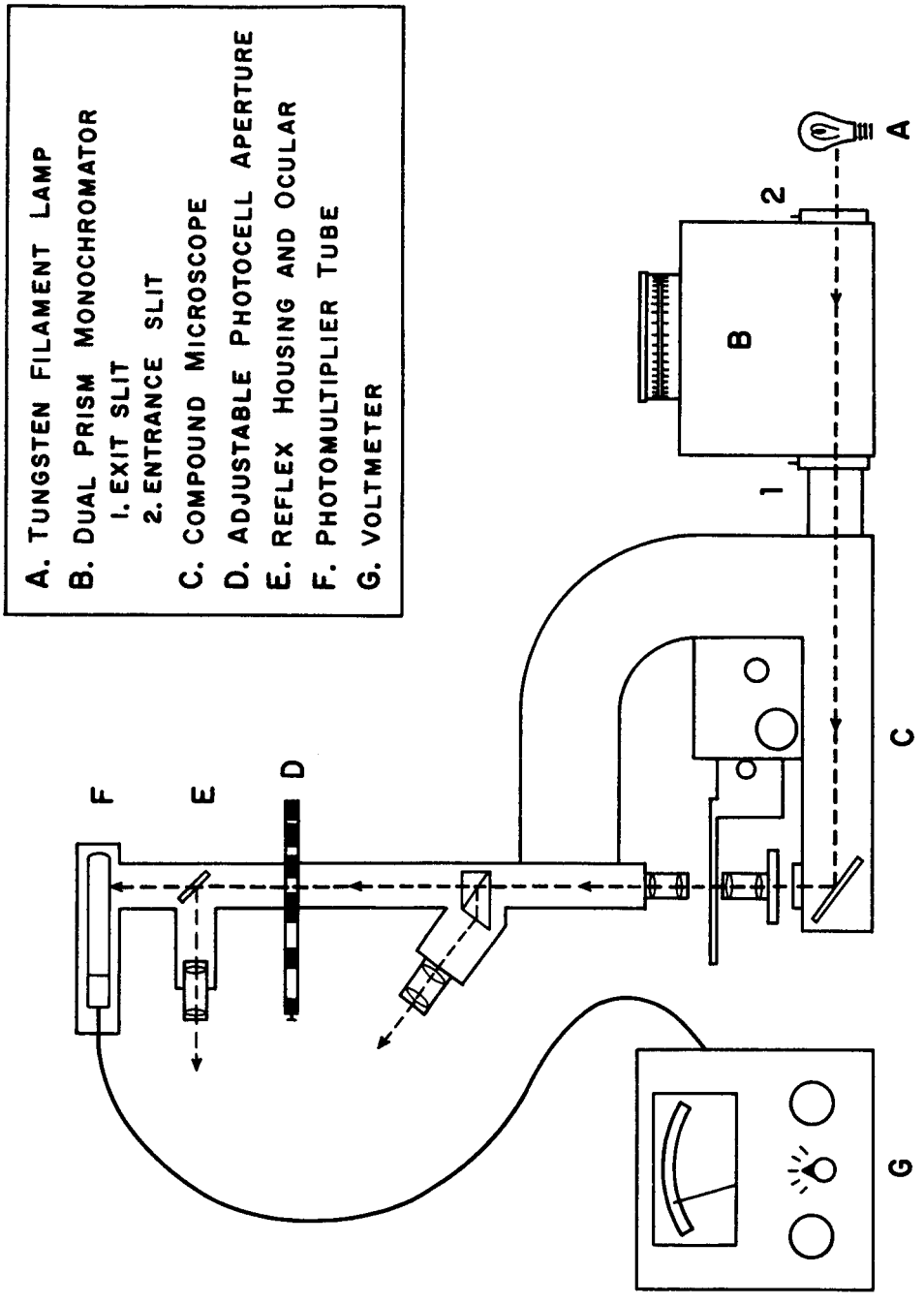

FIG. 2. DIAGRAM OF MAJOR COMPONENTS OF LEITZ MICROSPECTROPHOTOMETER.

the monochromator were set at 0.8 mm regardless of the wavelength used to illuminate the specimen. For this setting the spectral half-width is 8 mμ at a wavelength setting of 400 mμ and 48 mμ at a setting of 700 mμ.

Absorption measurements of stained pituitary cells were made at 20 mμ intervals in the visible range of the spectrum between 400 mμ and 700 mμ. At each wavelength transmission through the specimen (I_s) was recorded. The microscope slide was moved then to an immediately adjacent "blank" area of the slide, and transmission through the slide, mounting medium, and cover glass (I_o) was determined. The ratio of these readings (I_s/I_o) times 100 gave the percentage transmission (% Trans.) for the specimen at that wavelength. This calculation was repeated for each wavelength used. The procedure used to obtain a complete spectrum involved taking a series of readings with the specimen in position under the photocell after which the corresponding "blank" values were obtained. All percentage transmission (% Trans.) values were converted to percentage absorption (% Abs.) by subtraction from 100 (100 - % Trans. = % Abs.). The absorption spectra were plotted using percentage absorption as the ordinate and wavelength (20 mμ intervals) as the abscissa.

Mean optical density values (O.D. = $\log_{10} I_o/I_s$) were determined for the maximum absorption wavelengths (400 mμ for acidophil cells and 560 mμ for both basophil cell types) of each chromophil cell type in control and altitude-exposed rats. Quantitative comparisons of intracellular hormone concentrations following hypoxia exposure

were based upon these values rather than the mean percentage absorption data.

D. Statistical Analysis of Data.

For the cell count data standard deviations (σ) and standard errors (SE) are calculated for the means of each cell type in the pars distalis of control and every group of altitude-exposed rats. All reported results are expressed as the mean $\pm$ SE unless otherwise indicated. Significance of differences in the mean values is determined using the null hypothesis with Student's t-test employed to calculate probability (P) levels (77).

The microspectrophotometric data are treated in a similar manner. Mean percent absorption or optical density values and total range are determined for each cell type at all wavelengths in every group of animals. Standard deviations and standard errors of the mean at selected wavelengths (400, 480, 560, 660 mμ) are calculated for control animals and each group of altitude-exposed rats.

RESULTS

The results of this investigation are presented under four subheadings: A. Identification of anterior pituitary cells in tissue sections, B. Changes in rat pituitary weight in response to altitude exposure, C. Effect of altitude upon relative abundance of chromophobes, acidophils and basophils and D. Altitude-induced changes in anterior pituitary histophysiology demonstrated by cytophotometry. Although the first of these four does not constitute a definitive study, the data are instrumental in the pursuit and interpretation of the latter three subinvestigations, which together form the major thrust of the work.

Graphic and tabular summations are employed in the presentation of results, while the specific data from which these are prepared are included in Appendix B.

A. Identification of Anterior Pituitary Cells in Tissue Sections.

The application of five well-known staining procedures to formalin-fixed rat anterior pituitary tissue produced the results given in Table 1. Each procedure was selected originally for its reported ability to simultaneously differentiate several distinct pituitary cell types. Comparison of actual staining results with those results reported in the literature provided a basis for assessment of the utility of each stain for elucidation of pituitary structure and function during altitude acclimation.

Table 1. Anterior pituitary staining: A comparison of reported and actual results from five common stain procedures.

Stain Technique	Cell Types	Reported Results	Actual Results
P.A.S., methyl blue, Orange G (Wilson and Ezrin, 1954)	chromophobes	unstained	unstained
	acidophils	yellow to orange	orange
	thyrotrophs	red to magenta	red to magenta
	gonadotrophs	purple to blue	red to magenta
Aldehyde Fuchsin, Orange G	chromophobes	unstained	unstained
	acidophils	orange	orange
	thyrotrophs	deep purple	unstained
	gonadotrophs	unstained	unstained
P.A.S., Aldehyde Thionine, Luxol Fast Blue (Paget and Eccleston, 1960)	chromophobes	unstained	unstained
	acidophils	intense blue-green	unstained
	thyrotrophs	dense blue-black	purple
	gonadotrophs	red to magenta	purple
P.A.S., Alcian Blue, Picric Acid (Mowry, (1960)	chromophobes	unstained	unstained
	acidophils	yellow	yellow
	thyrotrophs	purple to blue	red to magenta
	gonadotrophs	red to magenta	purple to blue

None of the stain procedures yielded results identical to those reported when applied to the pituitary tissue used in this investigation. At the same time, partial staining was present in every case. Differentiation of chromophobe, acidophil, and basophil cells was accomplished with relative ease. These were best demonstrated by the Mowry technique and only slightly less well by the Wilson and Ezrin, and Elftman stains. Chromophobes were reported as unstained with all five procedures since they always displayed minimal amounts of non-specific staining. Acidophil cells stained equally well with orange G or picric acid, but the yellow acidophils following Mowry's stain contrasted more sharply with other chromophil cell types than when stained with orange G. Basophil cells stained clearly with the periodic acid-Schiff reaction, whereas aldehyde fuchsin was of no value in the differentiation of basophils in the pituitary tissue used in this investigation.

Of the staining techniques examined in this preliminary portion of the study, Mowry's P.A.S., alcian blue, picric acid combination produced the best separation of cell types. Four distinct types were noted and were designated as chromophobes, acidophils, basophil cells-type A, and basophil cells-type B. Further study of the distribution within the anterior lobe and individual cellular structure permitted identification of type A basophils as gonadotropic cells and type B basophils as thyrotropic cells. Gonadotropic basophils were colored with both the P.A.S. and alcian blue components of the stain and appeared purple, while thyrotropic basophils bound only the

products of the P.A.S. reaction and were red. Acidophil cells were yellow and chromophobes displayed either very little staining or were green in appearance. In all cases the stained cytoplasmic granules were employed to determine the category to which individual cells belonged.

On the basis of the clarity of separation of cellular types following repeated applications of the P.A.S., alcian blue, picric acid stain to the rat anterior pituitary, no further study of staining procedures was deemed necessary, and this became the stain of choice for tissue sections employed in the further pursuit of the major problem. A noteworthy result of this portion of the investigation was that identical staining reactions were visible in all rat anterior pituitary tissue regardless of the occurrence or duration of hypoxia. Thus, isolated microscopic fields displayed the same general appearance in both control and altitude-exposed pituitary sections.

B. Changes in Rat Pituitary Weight in Response to Altitude Exposure.

The effects of altitude exposure upon the weight of the intact pituitary (anterior and posterior lobes) are summarized in Figure 3 and Table 2. No changes in the weight of fixed glands occur during the first two days of decompression; however, seven days of continuous exposure to simulated altitude results in a significant ($P = .01$) loss in pituitary weight from a mean value of about 17 mg to about 15 mg. This loss persists, since the weight of the pituitary following 30 days of exposure is no different from that of rats exposed for seven days.

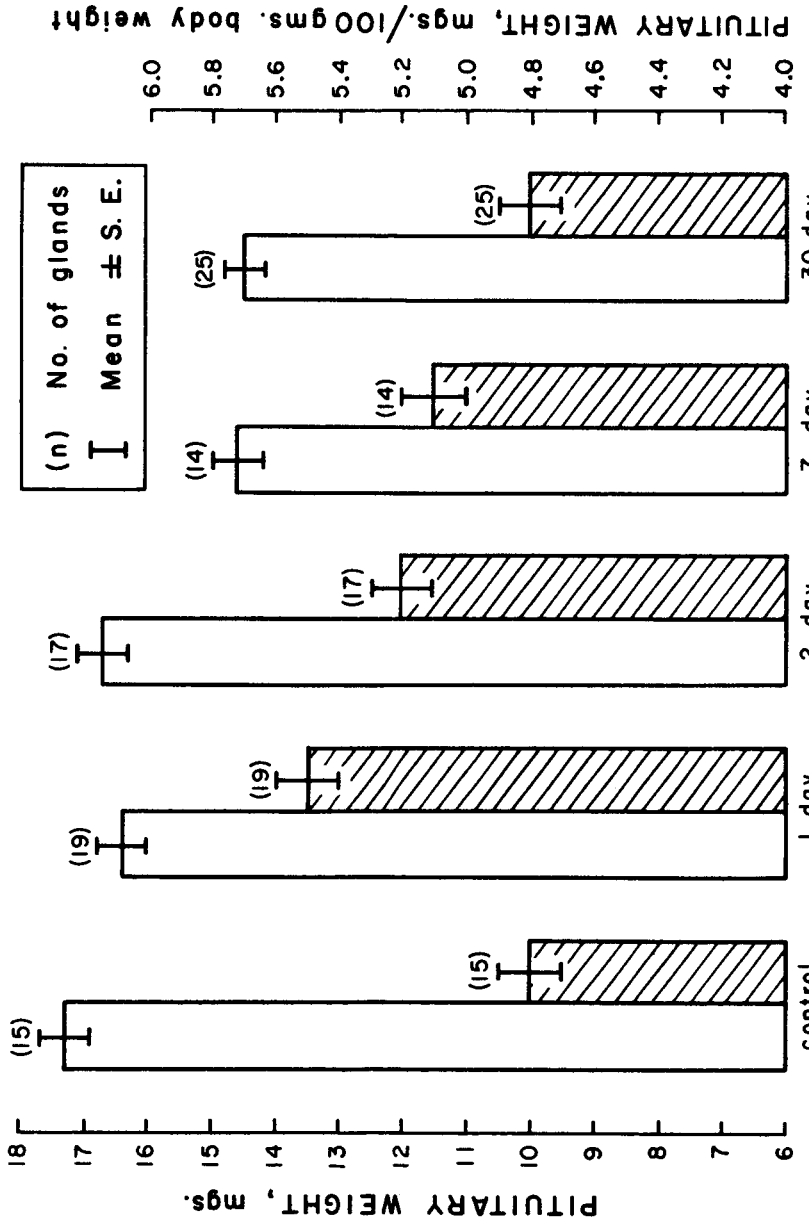

FIG. 3. ACTUAL AND RELATIVE (hatched) PITUITARY WEIGHT CHANGES DURING ALTITUDE EXPOSURE.

Table 2. Gross and relative pituitary gland weight changes and whole body weight adjustments in male rats exposed to simulated high altitude.

Exposure (380 mm Hg)	Date Placed in Chamber	Date of Sacrifice	Wt at Onset of Study (g)	(n) at Onset	Wt at Sacrifice (g)	(n) at Sacrifice	% Wt Change	Wt Fixed Whole Pituitary Gland (g)	Wt Pituitary/100 g Body Wt
Ambient pressure controls		11/20/62	258 ± 3	15	360 ± 7	15	+ 39.5	17.3 ± .4	4.8 ± .1
1 day exposed	11/19/62	11/20/62	342 ± 3	20	302 ± 3	19	- 11.7	16.4 ± .4	5.5 ± .1
2 day exposed	11/18/62	11/20/62	370 ± 5	20	327 ± 7	17	- 11.6	16.7 ± .4	5.2 ± .1
7 day exposed	11/13/62	11/20/62	337 ± 6	20	289 ± 6	14	- 14.2	14.6 ± .4	5.1 ± .1
30 day exposed	10/16/62	11/20/62	258 ± 3	25	300 ± 5	25	+ 16.3	14.5 ± .3	4.8 ± .1

n = number of male rats

The observation that there is a loss of body weight following altitude exposure (Table 2) makes it desirable to compute pituitary weight changes on a relative weight basis. The results of these calculations, in milligrams pituitary weight per 100 grams body weight, are included in Figure 3 (hatched bars), and show that the relative gland weight increases significantly ($P = .01$) from the control value of about 4.8 mg / 100 g to 5.5 mg / 100 g within one day of the onset of decompression. A less marked increase is present for two day-exposed and seven day exposed animals, although both mean values remain higher than controls, while prolonged exposure for 30 days shows a return of the relative pituitary weight to the control level.

C. Effect of Altitude upon Relative Abundance of Chromophobes, Acidophils, and Basophils.

Differential cell counts performed upon the pars distalis of rats exposed to simulated altitude for one, two, seven, and 30 days show that hypoxia exposure results in an alteration of the proportional number of the three chromophil types relative to that of the chromophobes. The specific results, expressed as percentage changes of each cell type in a unit area of anterior pituitary tissue, are summarized in Figures 4 and 5 and Tables 3 and 4 of Appendix B. Comparison of the cell count data from control rats and all groups of altitude-exposed rats taken together shows that hypoxia results in a significant ($P = .02$) increase in the number of all types of chromophil cells in the pars distalis. The detailed findings are summarized below.

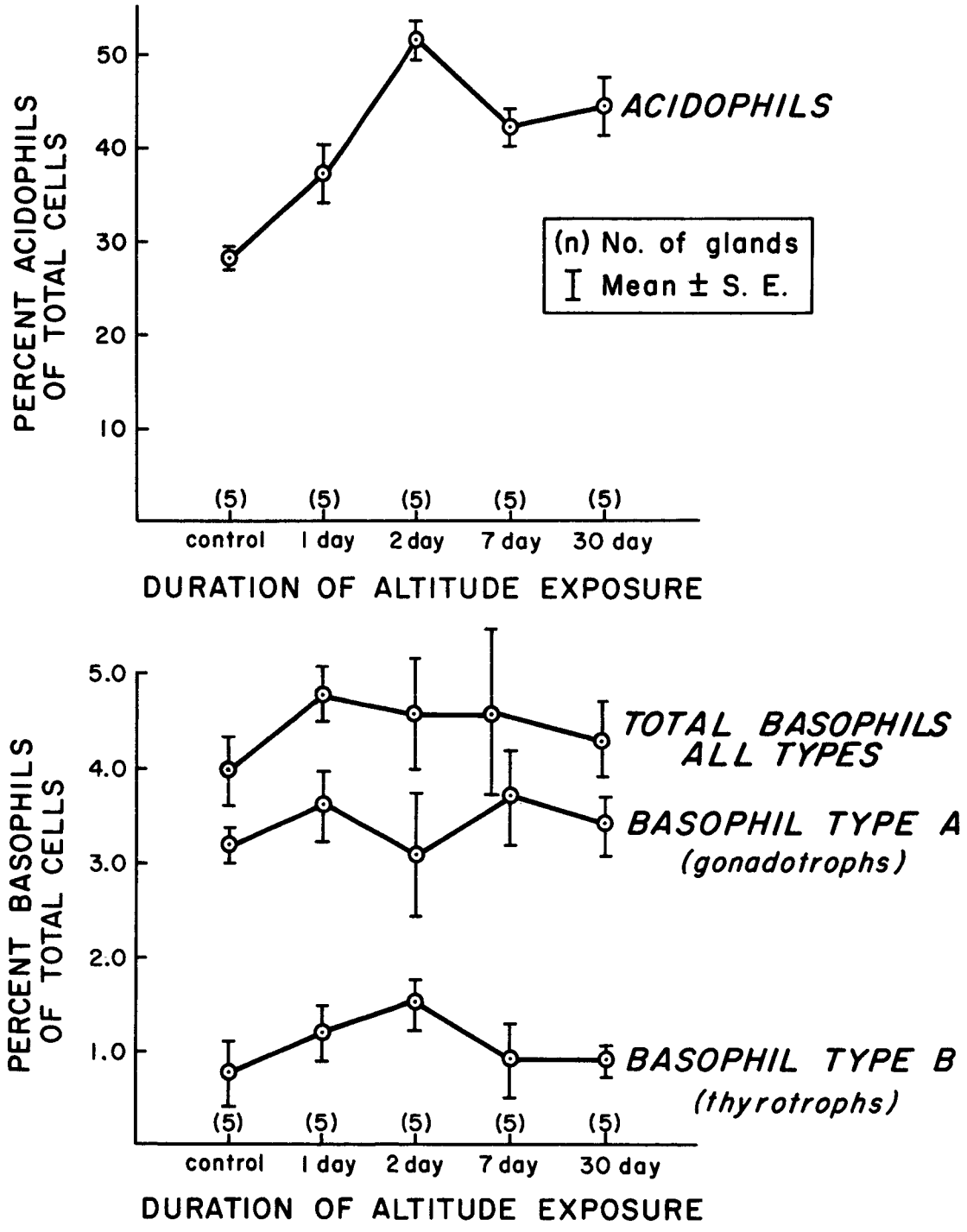

FIG.4. CHANGES IN RELATIVE NUMBER OF ANTERIOR PITUITARY CHROMOPHIL CELLS DURING ALTITUDE EXPOSURE.

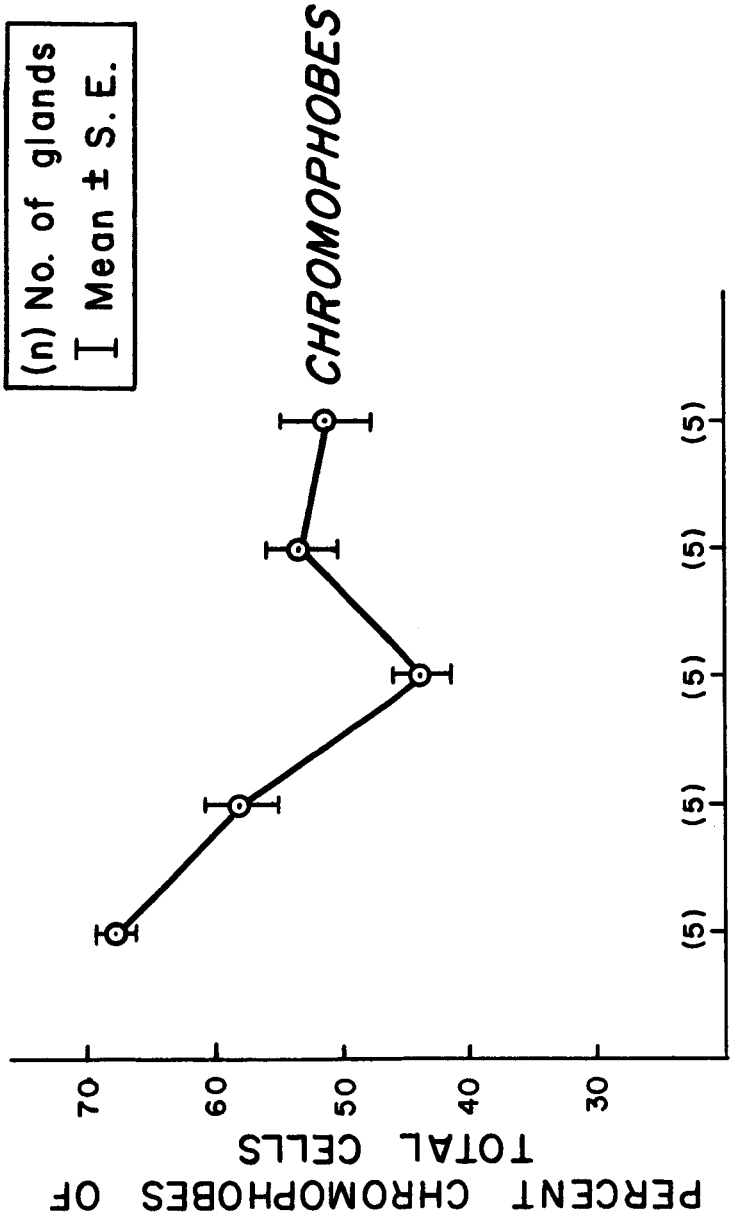

FIG.5. CHANGES IN RELATIVE NUMBER OF ANTERIOR PITUITARY CHROMOPHOBE CELLS DURING ALTITUDE EXPOSURE.

1. Acidophil cells. The first day of hypoxia exposure is marked by a sharp increase in percentage of acidophils ($P = .05$) from a control value of 28 percent to 37 percent (Figure 4). By day two 52 percent of all cells are acidophils. After seven and 30 days of exposure the relative abundance of acidophil cells remains higher than in ambient pressure control glands.

These results indicate that compensatory pituitary adjustments to simulated high altitude occur during the first few days of exposure, since the greatest departure from control value appears on the second day. Furthermore, the increase in acidophils is not a transient response since it persists throughout the 30 day exposure interval.

2. Gonadotropic and thyrotropic basophils. Hypoxia exposure results in an increase in the relative abundance of all basophil cell types ($P = .02$). This represents a significant increase on the first day of exposure. The percentage of total basophils remains slightly higher in two and seven day exposed rats but is not different from controls after 30 days of exposure (Table 3, Figure 4).

The results of differential cell counts for gonadotropic (type A) cells and thyrotropic (type B) cells are also included in Figure 4 and Table 4. Despite the high variability these data indicate that hypoxia exposure results in a transient increase in the relative number of thyrotrophs. For example, the combined data from one or two day exposed rats show a significant increase in type B basophils relative to that of the controls of the seven and 30 day exposed rats. The relative number of gonadotropic (type A)

basophils from hypoxic rats does not differ from that of ambient pressure controls. It should be noted however, that there is a markedly greater individual variability in the numbers of type A basophils in hypoxic rat pituitaries than in controls. Thus, it is evident that an alteration in the percentage of both basophil types contributes to the observed total basophil response pattern in hypoxic rats.

3. Chromophobes. Figure 5 illustrates the effect of altitude exposure upon the relative number of chromophobes. The data indicate there is a significant ($P = .01$) reduction in chromophobe cells in all altitude-exposed groups (Table 3). It can be seen that the pattern of chromophobe changes is exactly opposite to that of acidophils. Also the direction of changes is opposite that of basophils.

4. Additional observations. No evidence of mitotic activity is observed in any of the pituitary gland sections. Although the materials are not stained to show nuclear details, the cell nucleus is clearly visible, and structural changes associated with cell division would be evident. This observation indicates that the changes in relative abundance of the several cell types of the rat pars distalis in response to altitude exposure does not result from a true hyperplasia.

The presence of hyaline vacuoles in the cytoplasm of both types of basophil cell is observed to some extent in all groups of rats. Such vacuolization is especially prevalent in pituitary basophils of rats exposed to altitude for one, two, and seven days. Control

animals and rats exposed for 30 days have fewer vacuolated basophils. When vacuoles are present, the gonadotropic (type A) basophil closely resembles the classic castration cell, while thyrotropic (type B) basophils bear a striking resemblance to cells which appear following thyroidectomy.

D. Altitude-Induced Changes in Anterior Pituitary Histophysiology Demonstrated by Cytophotometry.

1. Confirmation of instrument sensitivity and response. Figure 6 shows the absorption spectrum of a Corning CS1-60 didymium glass filter obtained with the Leitz microspectrophotometer (Table 5). These results illustrate both the sensitivity of the instrument at various monochromator exit slit widths and the accuracy of response as judged by comparison of the resultant spectrum with one provided by the manufacturer. No significant differences are visible with exit slit widths of 0.8 mm, 0.6 mm, and 0.3 mm, and agreement with the manufacturer's curve is good.

2. Spectral characterization of stains and pituitary cell types. To aid in the eventual spectral characterization of stained pituitary cells, it is desirable to relate spectral data of stained sections to the absorption spectra of the dyes used in the staining process. Figure 7 shows an absorption spectrum obtained with a Beckman DB spectrophotometer from dilute solutions of each of the three dyes employed. These dyes are alcian blue (C.I. No. 74240, Ingrain Blue 1), the colored form of fuchsin-sulfurous acid from Schiff's reagent, and saturated aqueous picric acid. Examination

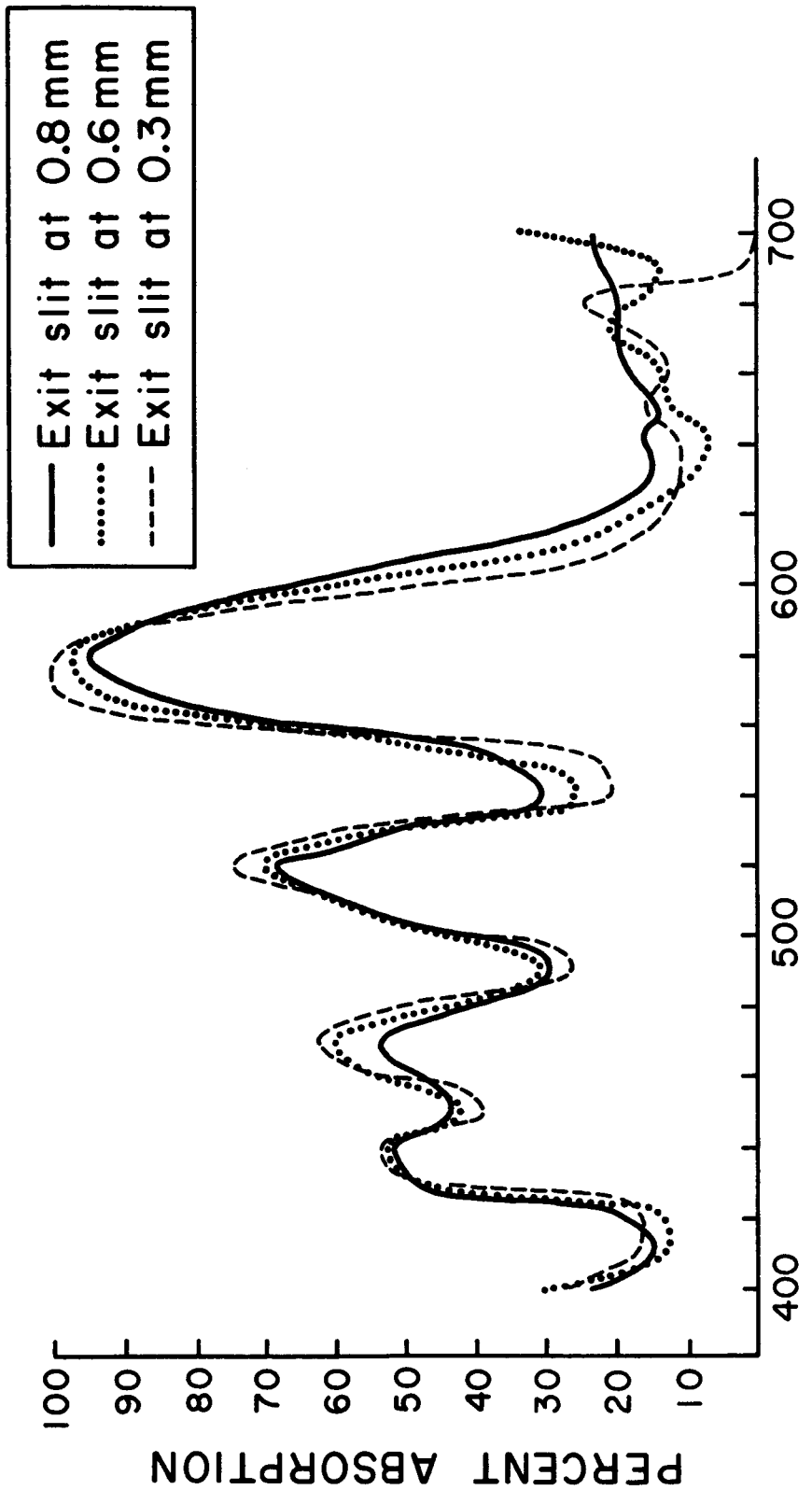

FIG.6. ABSORPTION SPECTRUM OF CORNING CS 1-60 DIDYMIUM GLASS FILTER OBTAINED WITH LEITZ MICROSPECTROPHOTOMETER.

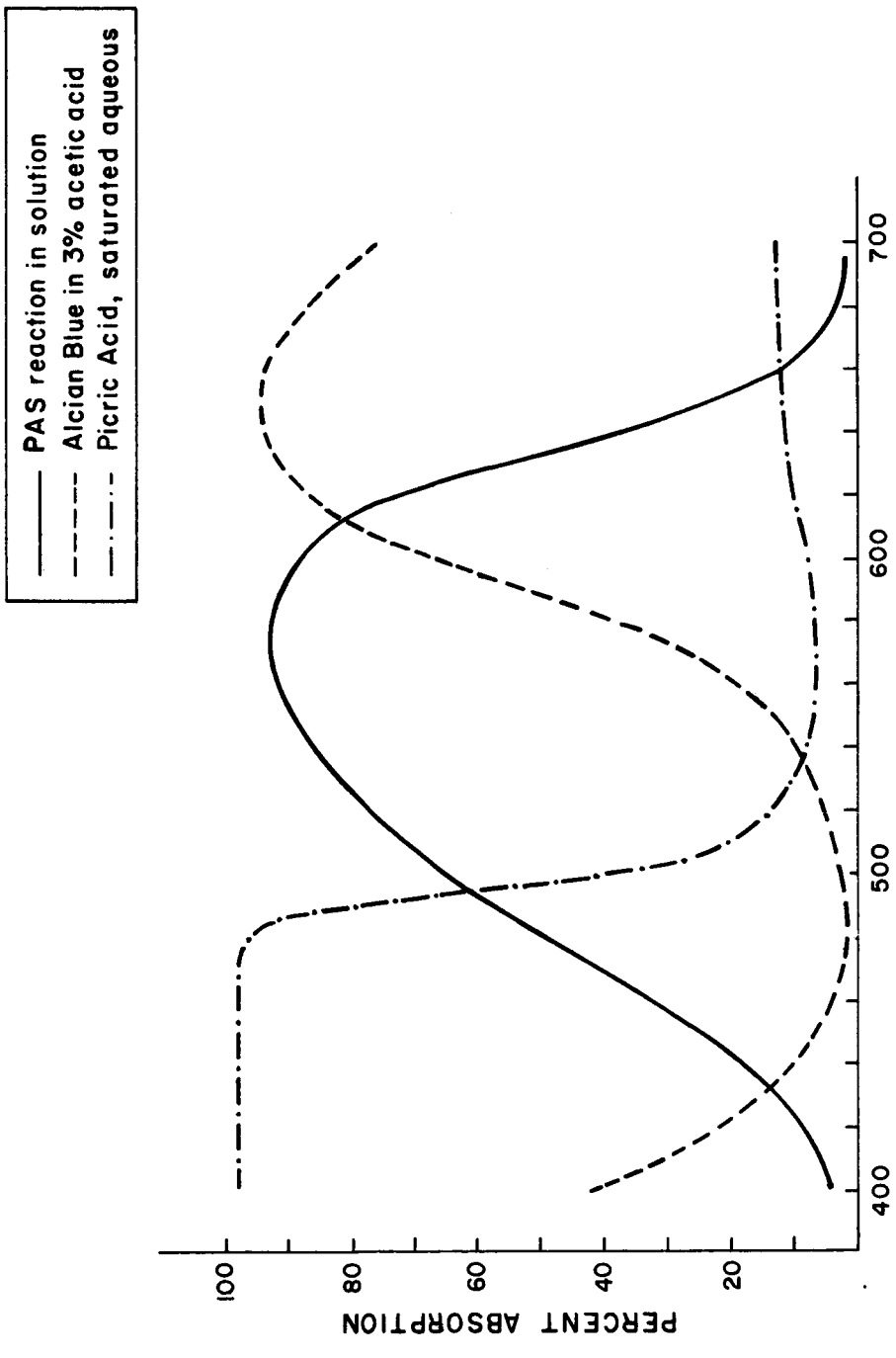

FIG. 7. ABSORPTION SPECTRA OF DYE SOLUTIONS USED FOR DIFFERENTIAL PITUITARY STAINING.

of these curves shows that each dye has an absorption maximum at a different wavelength.

The characteristic absorption spectra obtained from the cytoplasm of representative chromophil cells of the pars distalis are displayed in Figures 8, 9, and 10. Each of the curves results from a cytophotometric analysis of about 75 cells (three selected at random from each pituitary) judged to be of the same type. No distinction is made between cells from control rats and cells from altitude-exposed rats in the preparation of these curves. The general shape of the absorption spectrum for each cell type is indicated by the hatched area, which is determined, in turn, by the mean percent absorption at each 20 mμ interval in the visible portion of the spectrum. The width of the hatched curve represents the standard error of the mean, which is computed only at wavelengths of 400, 480, 560, and 660 mμ where maximum and minimum absorptions occur. The range of percent absorption values also is indicated at each spectral interval. Comparison of these absorption curves is facilitated by reference to such parameters as the regions of primary and secondary absorption, the absorption maximum, and the half intensity band width, which is defined as that spectral region which falls between the two points in the curve at which absorption equals one half the maximum primary absorption.

Examination of acidophil cells with the cytophotometer results in the characteristic absorption curve shown in Figure 8. Since the cells are colored with the picric acid component of the stain, the

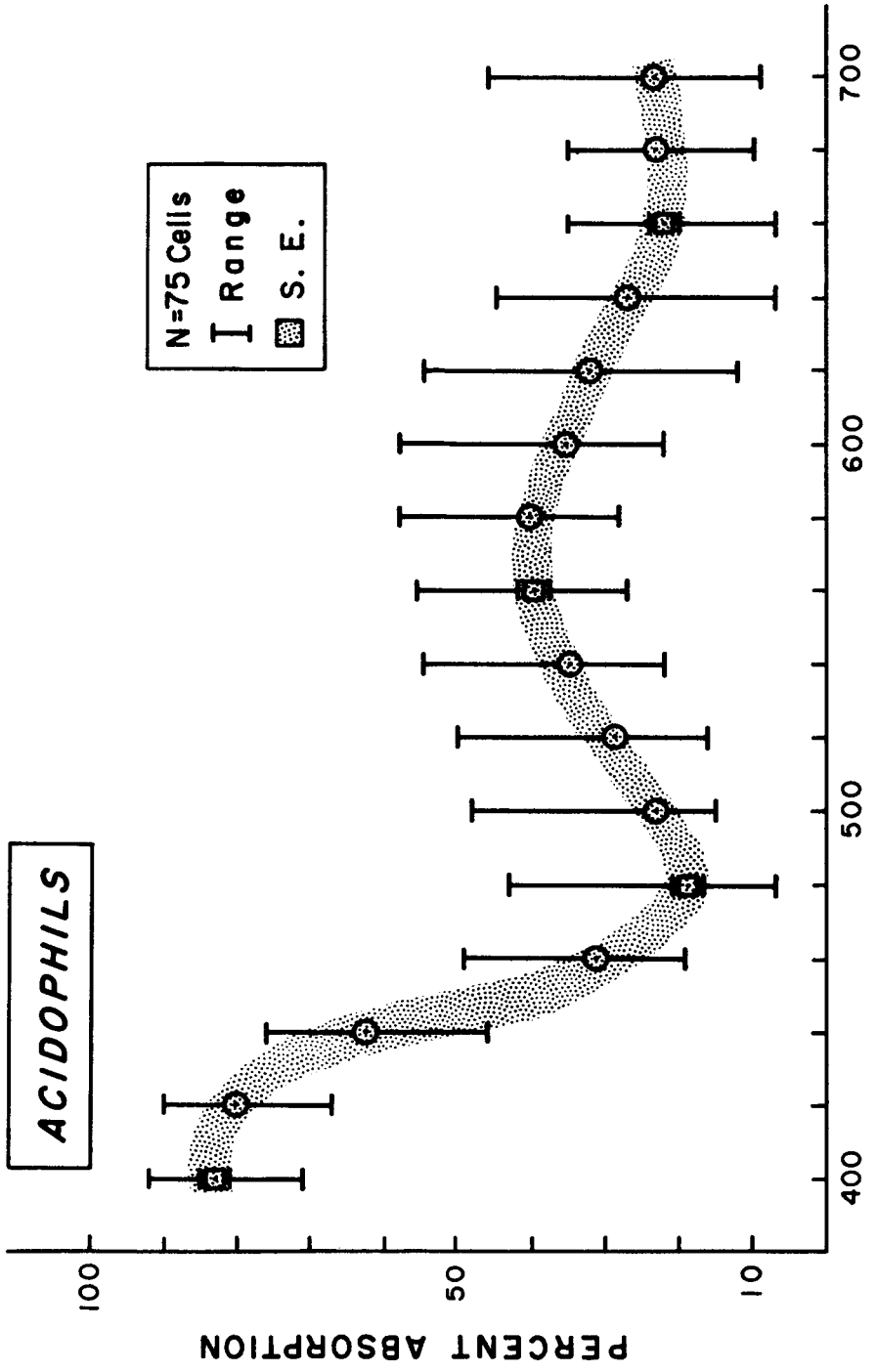

FIG. 8. MEAN ABSORPTION SPECTRUM FOR PITUITARY ACIDOPHILS (alcian blue, P.A.S., picric acid stain. This represents combined data from 25 control and altitude exposed rats).

primary absorption region is at a wavelength of 400-420 mμ. Some secondary absorption appears at 540-600 mμ. The absorption maximum is at 400 mμ. Table 6 presents the results of spectral characterization of acidophil cells in control rats and each group of altitude-exposed rats in addition to the mean percent absorption values and range for all examined acidophil cells. It is clear that the primary and secondary absorption regions, absorption maxima, and half intensity band widths are similar in all groups and do not change in response to altitude exposure. Thus, the observation that qualitatively similar acidophil staining occurs in all pituitary sections used in this investigation is confirmed.

Figure 9 shows the characteristic absorption spectrum obtained from gonadotropic (type A) basophil cells with the cytophotometer. The primary absorption region is between 540-580 mμ, with some secondary absorption appearing at 400 mμ. The primary absorption maximum is at 560 mμ, and the half intensity band width is from 480 mμ to 660 mμ. Examination of the absorption data (Table 7) obtained from pituitary gonadotropic cells of both control and altitude-exposed rats indicates that the binding of alcian blue and the colored products of the P.A.S. reaction occurs in a qualitatively similar manner in all cells of this type regardless of the occurrence or duration of hypoxia.

An absorption spectrum similar to that for gonadotropic basophils is obtained from thyrotropic (type B) basophil cells and is shown in Figure 10. The primary absorption region is again at

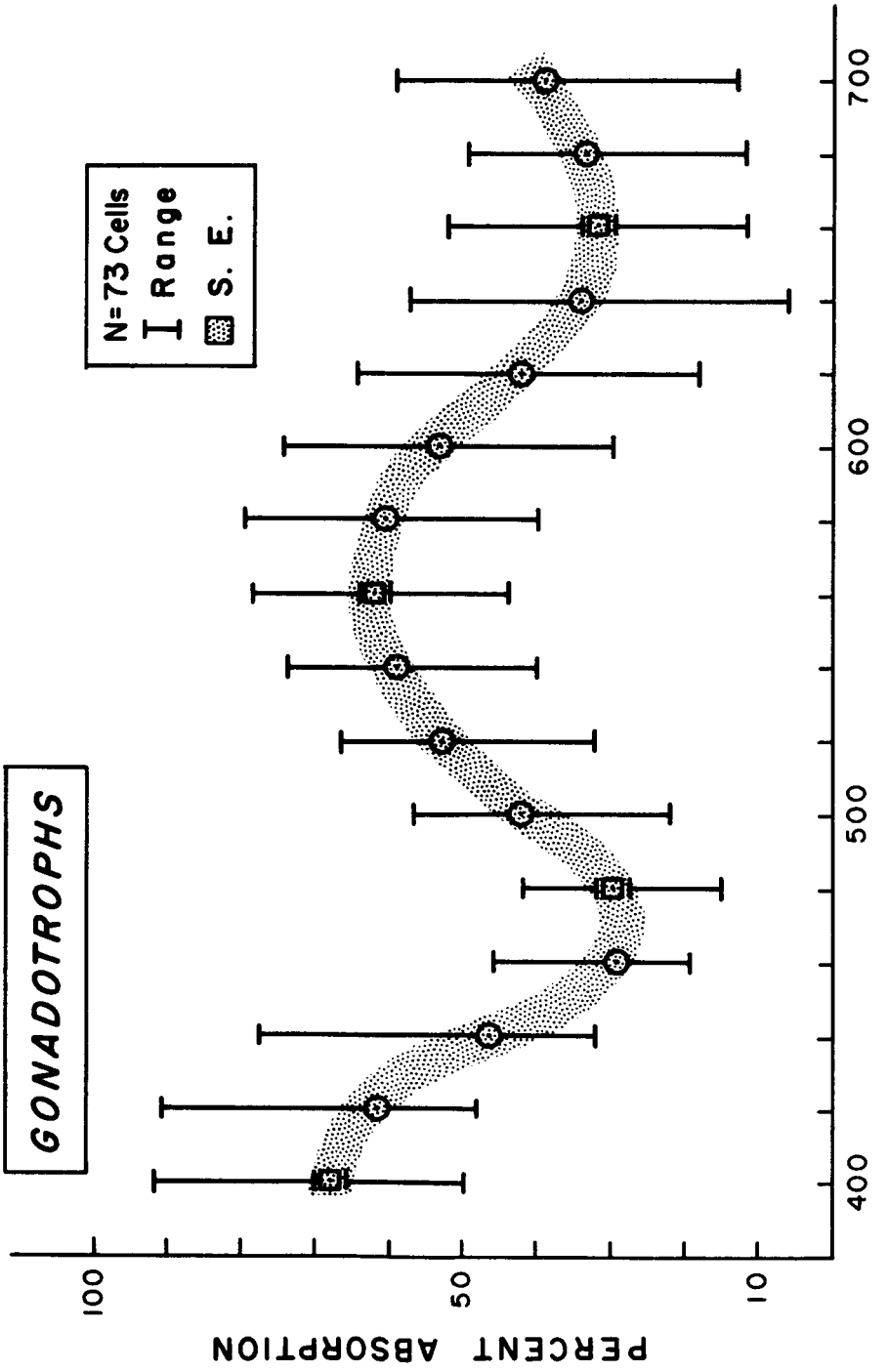

FIG. 9. MEAN ABSORPTION SPECTRUM FOR PITUITARY GONADOTROPIC BASOPHILS (alcian blue, P.A.S., picric acid stain). THIS REPRESENTS COMBINED DATA FROM 25 CONTROL AND ALTITUDE EXPOSED RATS

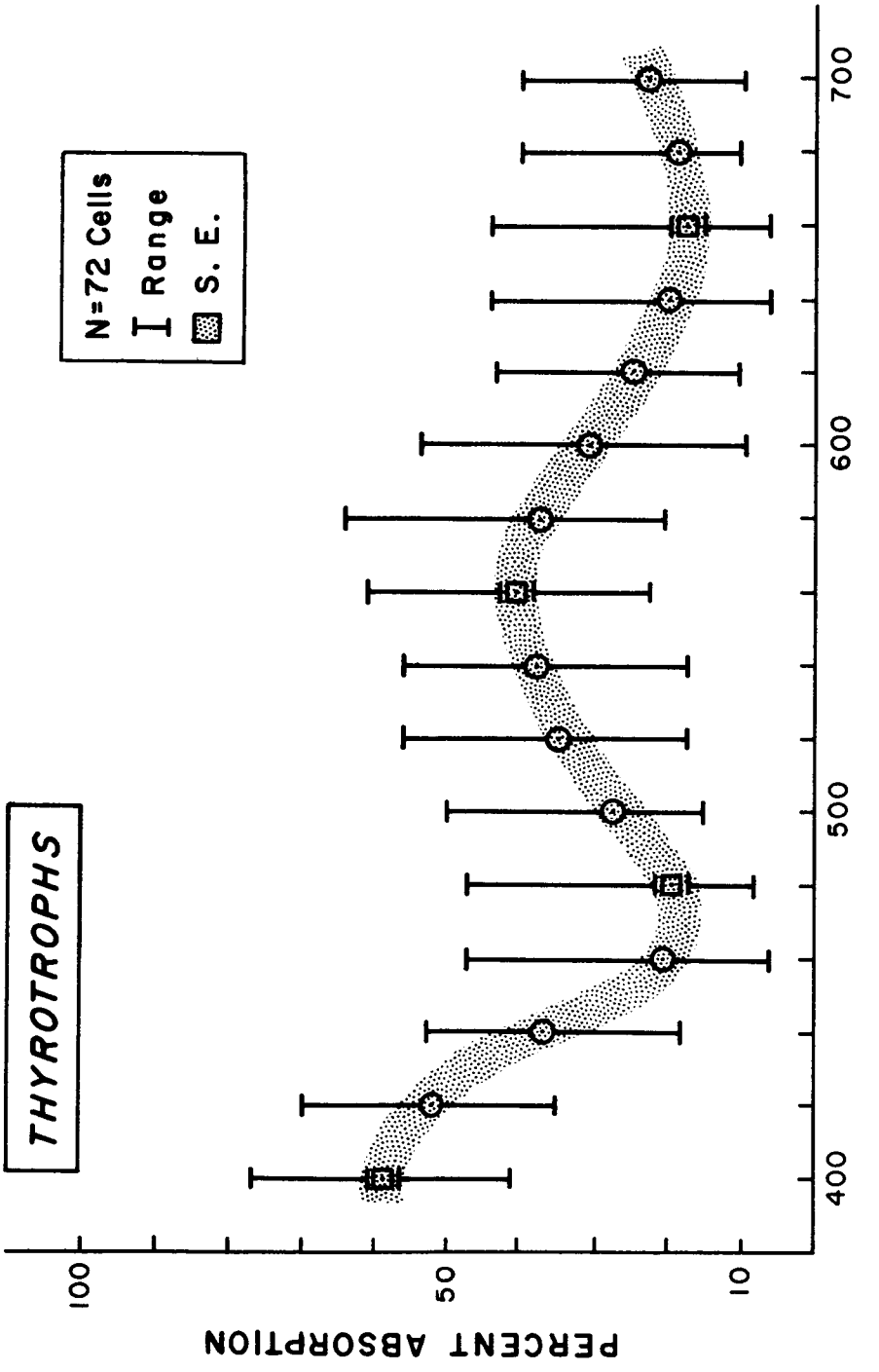

FIG.10. MEAN ABSORPTION SPECTRUM FOR PITUITARY THYROTROPIC BASOPHILS (alcian blue, P.A.S., picric acid stain). THIS REPRESENTS COMBINED DATA FROM 35 CONTROL AND ALTITUDE EXPOSED RATS.

540-580 mμ, with some secondary absorption at 400 mμ. Maximum absorption occurs at 560 mμ, and the half intensity band width is 480 mμ to 640 mμ. Comparison of absorption data (Table 8) from control and altitude-exposed rat pituitary cells indicates that the qualitative staining of thyrotropic basophil cells is also unaffected by altitude exposure.

The high variability (range) of the absorption data for all cell types at all wavelengths is due to the fact that no two chromophil cells are in exactly the same stage of secretory activity at the time when the tissue specimen is secured. Thus, the results of cytophotometric characterization of cells from the pars distalis give some indication of the dynamic nature of secretion.

3. Relative concentration of intracellular hormones during acclimation to hypoxia. The relative quantity of intracellular hormone present in individual chromophil cells of each type in the pars distalis may be estimated by quantitative cytophotometric analysis. Hypoxia-induced changes are detected by comparison of mean optical density (O.D.) values obtained from control rat pituitary chromophil cells of each type and similar cells in every group of experimental animals. Measurements are carried out at the primary maximum absorption wavelength for each chromophil cell type. Concurrent cell staining, uniform section thickness, and identical area of spectral measurement assure the equivalence of intracellular hormone concentration and optical density values. Thus, the height in O.D. units of the primary absorption peak recorded from any cell

is an indication of the intracellular hormone concentration.

Mean optical density values, calculated from spectral data obtained at 400 mμ from acidophil cells in control and altitude-exposed rat pituitaries, are displayed in Figure 11 and Table 9. The results indicate that intracellular hormone concentration rises sharply in response to the onset of hypoxia and remains significantly above control values in all experimental groups. Two and seven day exposed rats show somewhat smaller O.D. values for acidophil cells than animals exposed for only one day, while the greatest change is observed after 30 days of continuous hypoxia when individual cells display more than a tenfold mean increase in intracellular hormone concentration. The significance of these changes in relation to exposure duration is not apparent, although it is clear that acidophils of the pars distalis respond rapidly to hypoxic conditions.

Figures 12 and 13 and Table 9 illustrate related results obtained respectively from gonadotropic and thyrotropic basophil cells of the pars distalis in control and altitude-exposed rats. Mean optical density values are determined at 560 mμ for both cell types. Intracellular hormone concentrations increase significantly in the gonadotropic basophils of all experimental animals. The most striking changes occur within the first two days following the onset of hypoxia, while prolonged exposure produces further increases to a maximum value after 30 days. In contrast, thyrotropic basophil cells show increased intracellular hormone concentrations resulting from altitude exposure, but the amplitude of change is not as great and

the effects of exposure duration are less clear. One and 30 day exposed rats show the greatest increases, while the two and seven day experimental groups exhibit intermediate levels of intracellular thyrotropin.

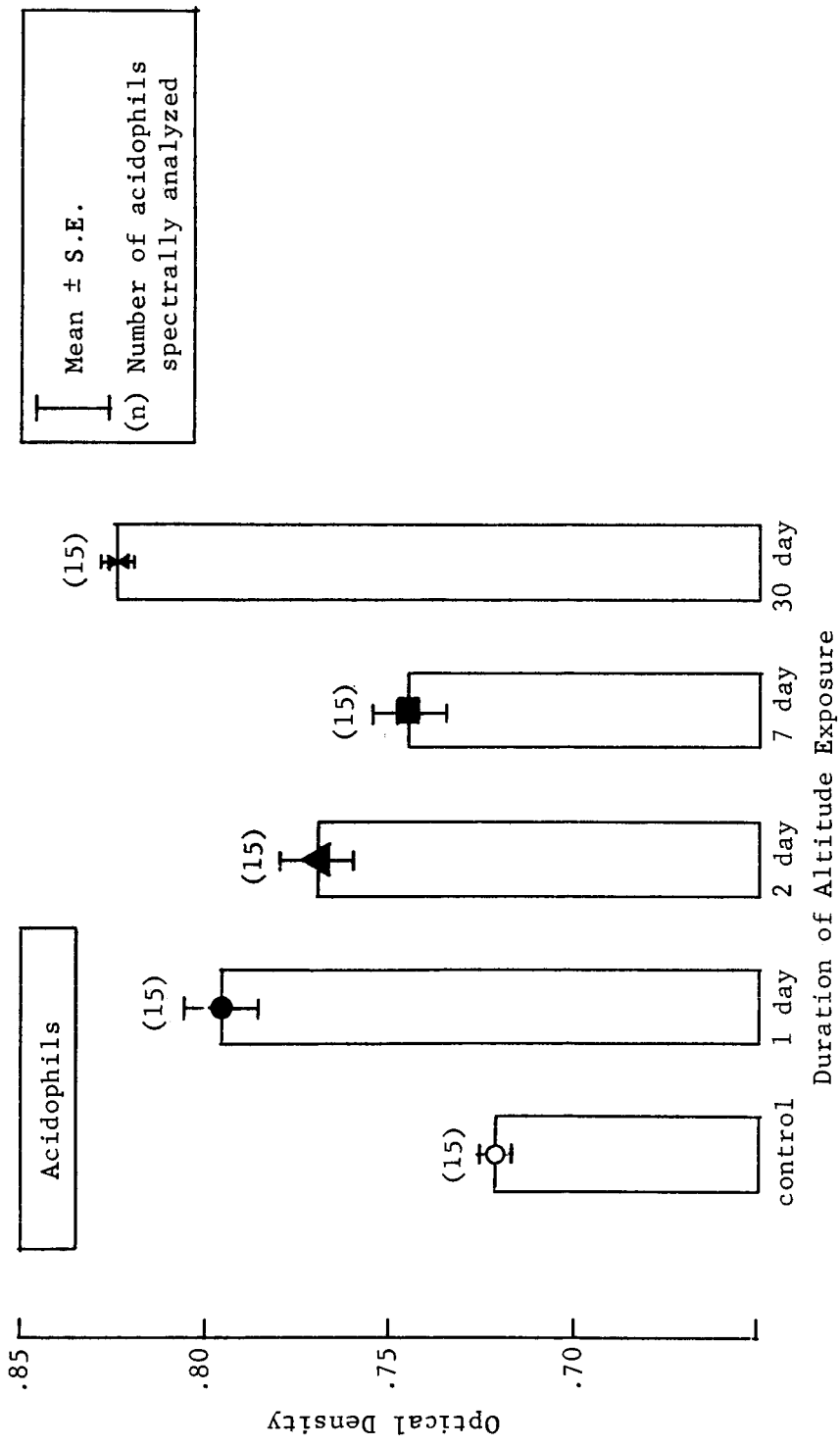

Figure 11. Effect of altitude exposure upon intracellular hormone concentration in pituitary acidophils (optical density at 400 mμ).

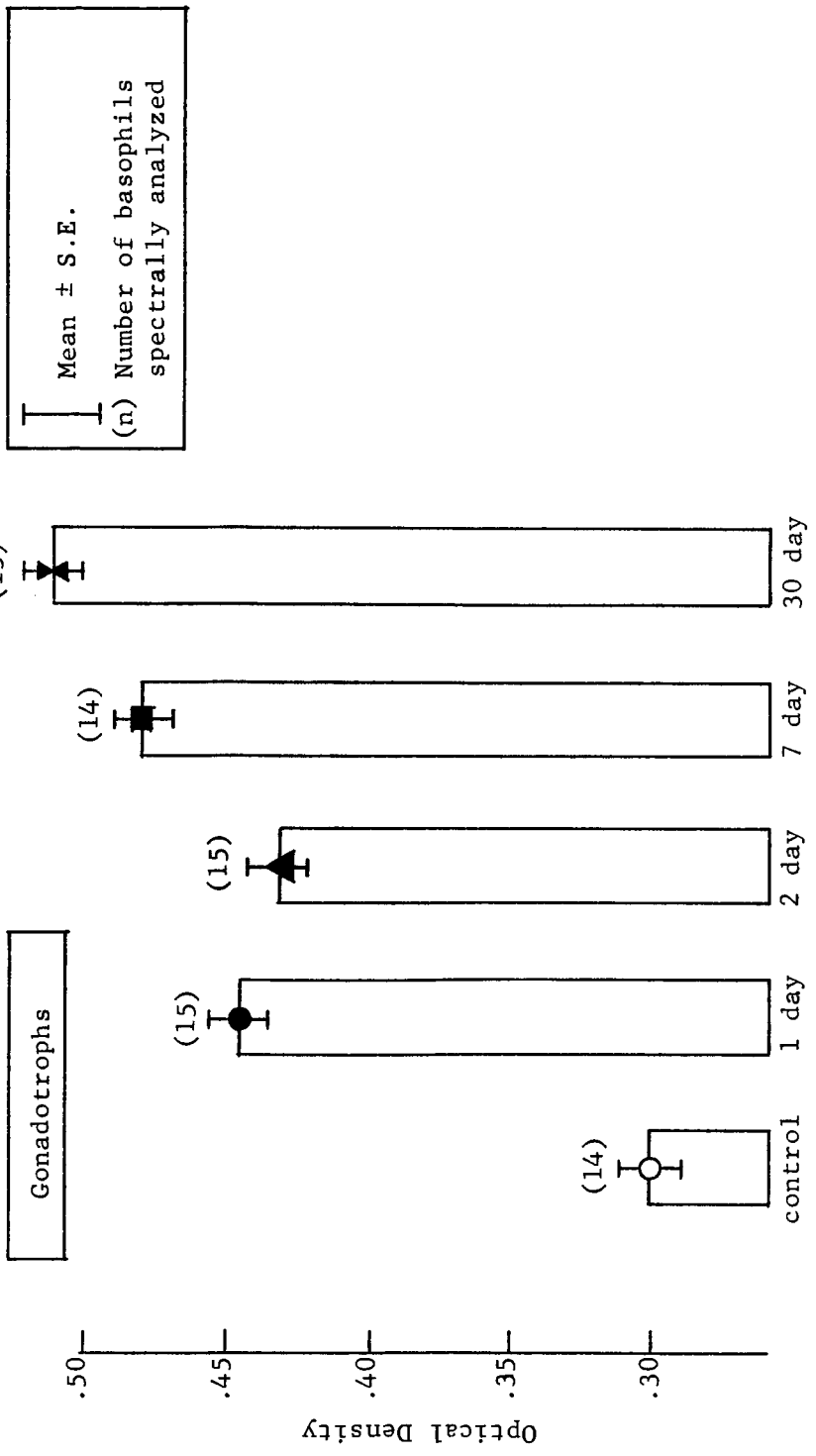

Figure 12. Effect of altitude exposure upon intracellular hormone concentration in pituitary gonadotropic basophils (optical density at 560 mμ).

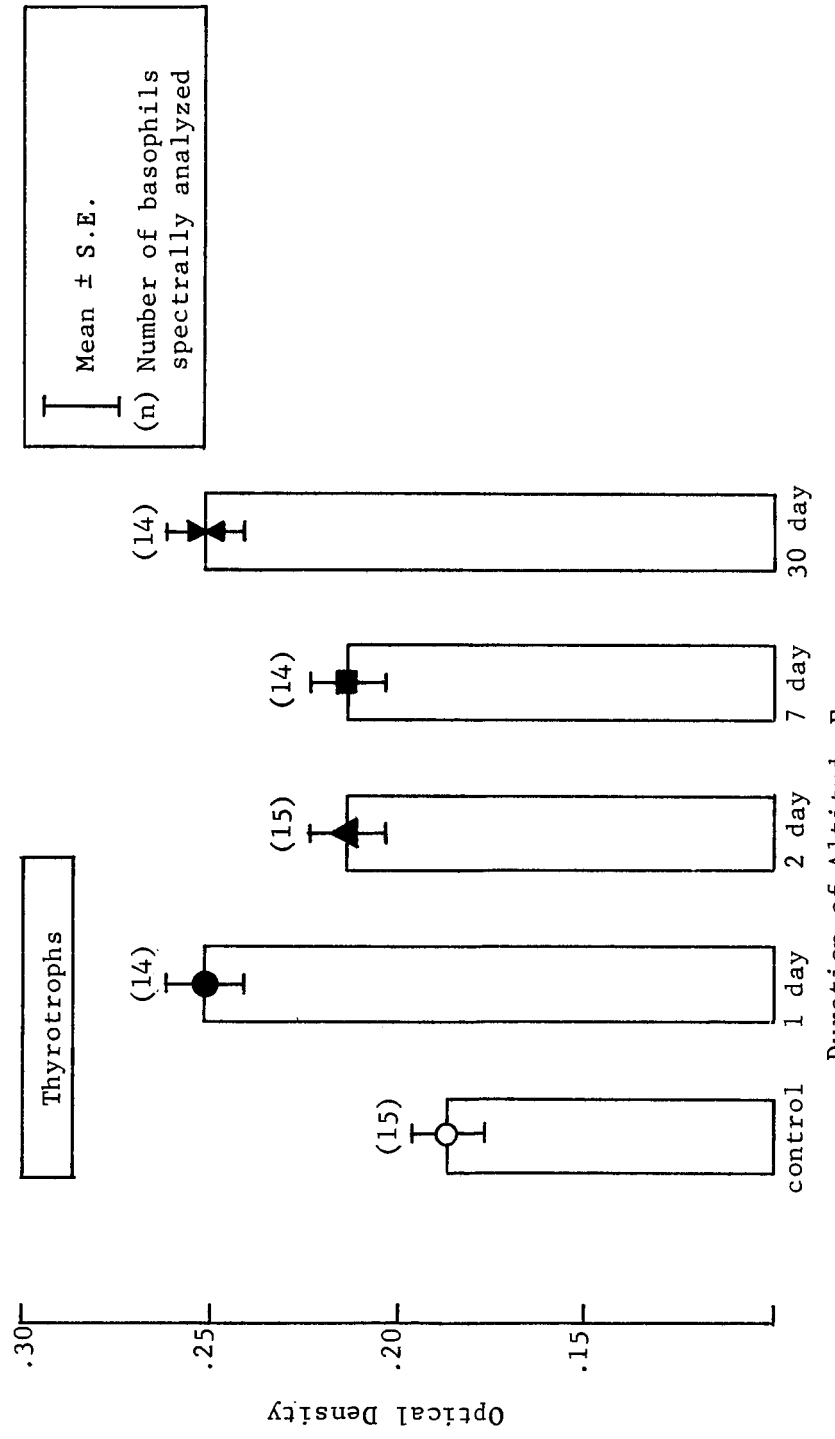

Figure 13. Effect of altitude exposure upon intracellular hormone concentration in pituitary thyrotropic basophils (optical density at 560 mμ).

DISCUSSION

It is evident that the anterior pituitary participates in the acclimation of the male rat to a simulated high altitude of 18,000 feet. Changes in the relative number of pituitary chromophil cells, in whole gland weight, and in the relative quantity of intracellular hormone are demonstrable within 48 hours or less of the onset of altitude exposure. Taken together, all of the data indicate that anterior pituitary hormones accumulate within the tissues of the pars distalis following hypoxia exposure, although it is not clear whether this results from increased hormone synthesis, decreased hormone secretion, or a combination of both.

Since the study of pituitary histophysiological changes is greatly facilitated by differential staining of tissue sections, initial discussion centers on the problems which surround the demonstration and identification of cell types in the rat pars distalis.

Several stains appear to be of value in the differentiation of anterior pituitary cells. Of these, the P.A.S. technique appears to be the most reliable and has the added advantage of being histochemically specific for the aldehydes which are produced from tissue polysaccharides by periodic acid oxidation of 1, 2 glycol groups (47). The results of the present study emphasize the value of this stain for the demonstration of pituitary basophil cells. Observed morphological features and distributional patterns of P.A.S.-positive cells

in pituitaries of both normal and altitude-exposed rats are similar and in close agreement with those reported by Purves and Griesbach (67). Thus, thyrotropic basophils (type B) are found only in the central areas of the gland, occur in groups, are irregular in shape, and are rarely adjacent to blood vessels, while gonadotropic basophils (type A) are distributed both in the central and peripheral areas, are round or oval in outline, and frequently make contact with elements of the circulatory system.

Aldehyde fuchsin is reported by some workers as a good selective stain for thyrotropic basophils (17, 32). Results of the present study show that aldehyde fuchsin fails to differentiate the two types of basophils. This is in keeping with the erratic performance of this dye reported by others (1, 17, 33, 57) and may be due, in part, to the use of formalin as a fixative. Elftman recommends the use of chrome-alum as a fixative for best results with aldehyde fuchsin staining (16, 17).

Alcian blue is also of value for anterior pituitary cytodifferentiation (1, 35, 48, 51). Mowry (53) reports that acidic tissue polysaccharides are stained with dye solutions of low pH, although the mechanism of staining remains unknown, and there is need for dye standardization (13). The results of the present investigation in which differentiation of gonadotropic basophil cells is enhanced by their tendency to bind alcian blue are in sharp contrast to those reported for mouse (48) and human (1) tissue where thyrotropic cells are stained. This may reflect interspecific

variability or may simply result from the present stain procedure which does not include tissue oxidation by performic acid prior to alcian blue staining. It is noteworthy that altitude exposure has no apparent effect upon the tinctorial affinity of pituitary cells for alcian blue.

Although no histochemical specificity can be ascribed to the picric acid component of the stain employed in this investigation, other acid dyes used to demonstrate acidophil cells, such as orange G (88), luxol fast blue (57), and light green SF (32), are not superior in this respect. As with all other stain components, the binding of picric acid by acidophil cells remains unaltered by altitude exposure.

Despite the success of these several coloring agents for differentiating the cellular composition of anterior pituitary tissue, the inconsistencies in staining intensity, distribution, and performance in the hands of different investigators emphasize the need for procedural standardization (84). A major, though supplemental, contribution of the present study is the demonstration that the cell types of the rat anterior pituitary, once stained, can be characterized successfully on the basis of their respective absorption spectra. Thus, comparative studies of staining intensity, dye-tissue complexing, multiple dye binding, and dye distribution become feasible. Cytodifferentiation in heterogeneous tissues such as the anterior pituitary is greatly facilitated and need no longer be done on a wholly subjective basis, since the "appearance" of the

individual cell also may include objective cytophotometric identification.

An accurate assessment of the effects of simulated high altitude upon the rat anterior pituitary necessitates direct visual observation of pituitary cells to corroborate the wealth of indirect data which indicate the presence of acclimating pituitary mechanisms. For example, the endocrine epithelium of the pars distalis is known to be engaged in the synthesis and release of hormones, and alterations in one or both of these functions constitute the only mechanisms by which the pituitary can contribute to acclimation processes. Direct observation of pituitary cells should demonstrate one or more of the following: 1. a change in the number of specific hormone-producing cells of each type, 2. alterations in the synthesis or storage of specific hormones as reflected in the intensity of cytoplasmic granulation, and 3. an increase or decrease in hormone secretion as reflected in depletion of granulation and cytoplasmic vacuolization. The net effects of the latter two cytoplasmic changes would be indicated by the relative amount of stained intracellular hormone present in individual cells of a particular type during acute and chronic hypoxia exposure.

Differential cell counts obtained from histochemically stained pituitary sections support the conclusion that observable changes do occur in the number of each type of chromophil cell present. More specifically, altitude exposure results in an increase in the frequency with which the three types of chromophil cells appear.

This effect is most evident in the acidophil cell population and to a lesser degree in the gonadotropic and thyrotropic basophils. Furthermore, the number of chromophil cells of each type present in control rats, expressed as a mean percent of total cells, is in good agreement with reported results from other studies not related to altitude acclimation (42, 49).

Because the distribution of chromophil cell types within the rat pituitary conforms to a definite pattern (67), differential counting of random microscopic fields does not yield a true picture of changes in cell frequency. Failure to observe this feature of pituitary gland organization may explain the limited success of early attempts to correlate count data with functional changes (28, 63). The count procedure herein described is designed to minimize errors due to non-random cell distribution.

Attempts to demonstrate mitotic activity in the pars distalis of the male rat show that this is a very rare event (14, 50), and no mitotic figures are observed in the pituitary sections employed in the present study. Thus, the apparent increase in chromophil cells of all types following hypoxia exposure does not represent a true hyperplasia. The clearest support for this conclusion comes from the cell count data for chromophobes which show a decrease in number paralleling the increases in the chromophil series.

In short, all of the cell count data indicate that intracellular hormone concentrations increase within individual anterior pituitary cells following hypoxia exposure. Reduction in the rate of cellular

hormone secretion is the most probable mechanism accounting for the observed changes. Increased synthesis of pituitary hormones cannot be ruled out, especially for ACTH (46), but seems unlikely, since lowered oxygen availability is known to inhibit protein synthesis in certain rat tissues (70). Finally, it is clear that in control male rats at least some pituitary chromophobes are hormone-secreting cells whose cytoplasm is depleted of secretion granules. This appears to be particularly true for the acidophil series which shows the greatest change in numbers of any chromophil cell type in the present study and is known to show great lability under experimental conditions other than altitude exposure (30, 42, 49).

The reported effects of simulated high altitude upon pituitary weight are conflicting. Although Moore and Price (52) and Marks et al. (46) assert that no significant weight changes occur, the present investigation shows a loss of gland weight during hypoxia exposure and supports the results of Gordon et al. (28). It seems clear that pituitary weight changes are associated with the general loss of body weight which results from interruption of normal patterns of food and water consumption during the acute stages of acclimation (54, 78, 83). Calculation of changes in relative pituitary weight show that this loss occurs more slowly in the tissues of this gland than in the body as a whole. This could reflect the increased blood flow reported for the pituitary under conditions of stress (26) or might be due to transient accumulation of intra-glandular secretory products, since histological observation reveals

no unusual accumulation of blood within the vascular spaces of the pars distalis.

The results of cytophotometric analyses lend a new degree of sophistication to the direct investigation of histological pituitary sections. This phase of the study involves the objective spectral characterization of respective cell types in heterogeneous tissues from different sources, and an assessment of hypoxia-induced changes in relative quantities of intracellular materials.

Application of cytophotometry to anterior pituitary tissues from control and altitude-exposed rats supports the following conclusions: 1. altitude exposure has no effect upon the spectral characteristics of individual chromophil cells when these are stained with alcian blue, P.A.S., and picric acid, 2. individual pituitary acidophil cells show an increase in dye-binding capacity as a result of altitude exposure, and 3. individual basophil cells of both the gonadotropic and thyrotropic series demonstrate an increased dye-binding capacity following exposure to simulated high altitude.

The spectral characteristics of dyes such as those employed in this study may be visualized by reference to the shape of the absorption curves obtained from dye solutions and from tissue components to which the dye is bound following staining (13). Cytochemical changes occurring in tissues as the result of an mental stressor such as hypoxia exposure may alter the dye-tissue complex and produce changes in the position and shape of dye absorption peaks. With the exception of alcian blue staining of

gonadotropic basophils, such changes are not observed in the present study, and absorption curves from respective chromophil cell types are similar to those which one would predict from observations of dye solution spectra. Furthermore, since absorption curves from any given chromophil cell type have the same general configuration for both control and all experimental animals, it is evident that no qualitative cytochemical alterations occur in the secretory products of respective hormone-producing cells as a result of altitude exposure.

Cytophotometric characterization of stained gonadotropic basophil cells raises some important questions concerning the extent to which these cells bind alcian blue, since only one major absorption peak, corresponding closely with that of the P.A.S. component of the stain, appears in spectra obtained from these cells. Either the major absorption of alcian blue in solution shifts to a shorter wavelength region of the spectrum when the dye is complexed with tissue polysaccharides, or the apparent color differences between thyrotropic and gonadotropic basophils depend entirely upon differences in P.A.S. stain intensity. Further study is needed to resolve this problem.

Although quantitative cytophotometric analysis is well established as a technique for measuring intranuclear DNA in Feulgen stained tissue sections (50, 62, 80), application of this method to stained cytoplasmic structures in ordinary histological preparations is still in its infancy. Effective application of this method depends upon elimination of sources of error such as

nonhomogeneous dye distribution, irregularities in section thickness, and improper instrument alignment and response. Of these three, the first is reported to be of only occasional importance (20), while the second is minimized in the present study by analysis of many cells always selected from different tissue sections. The third source of error is obviated by repeated daily checks of instrument alignment and of photocell response linearity.

The data obtained from quantitative cytophotometric analyses of pituitaries support the major conclusions concerning pituitary histophysiological changes during altitude acclimation based on cell count and weight change data. Specifically, cytophotometric examination of chromophil cells of each type in control and all groups of altitude-exposed rats shows that the relative quantity of intracellular hormone, as measured by an increase in P.A.S. staining intensity (21), rises within a short time in gonadotropic and thyrotropic basophil cells. Acidophil cells, which stain with picric acid, also show an increase in stain intensity and intracellular hormone content.

In summary, it is clear, from the combined results of cell counts, weight changes, and cytophotometric analyses of individual cells, that under conditions of simulated high altitude the rat anterior pituitary gland shows a net accumulation of hormone materials in all known hormone-producing cell types. This functional change manifests itself both as an increase in the proportionate number of a given type of pituitary chromophil

cell and as an increased cytoplasmic affinity for one or more dyes after differential staining. Thus, acidophil cells show a marked increase in number following hypoxia exposure and also display measurable changes in cytoplasmic hormone concentration, while gonadotropic and thyrotropic basophils appear in only slightly greater numbers and show substantial increases in relative amounts of intracellular hormone.

Pituitary adjustments resulting from exposure of male rats to simulated high altitude constitute a major factor in the attainment of the acclimated state. The findings of this investigation are in agreement with the hypothesis of Nelson (54) and others (34, 79), that pituitary TSH secretion may be slowed at the onset of hypoxia exposure thus contributing to the observed hypothyroid condition of experimental animals. In a similar manner, retention of anterior pituitary gonadotropins might contribute to the gonadal dysfunction which often accompanies exposure to altitude (83). Intracellular storage of growth hormone, as evidenced by an increased number of acidophil cells and greater concentration of intracellular hormone, could account for the reported reduction in growth rate in hypoxia-exposed rats (2), and may also produce effects upon the thyroid through synergistic action with TSH (82) and other unknown interactions which link changes in the acidophil cell with the thyroid (30, 42, 49).

It is of further interest to speculate whether the faulty lactation reported for female rats exposed to altitude (52, 83) may

result from the tendency of the anterior pituitary acidophil cells to retain greater than normal quantities of lactotropic hormone. The present study suggests the existence of such a mechanism.

This investigation does not resolve the mechanisms of pituitary-adrenal interaction which result from the stress of altitude exposure. The general retention of hormone products exhibited by pituitaries of hypoxia-exposed rats helps to explain the increased intrapituitary ACTH content reported by Marks *et al.* (46), but it does not provide an adequate explanation for the observed rise in circulating ACTH levels or increase in adrenocortical function (37) during the period of acclimation.

In conclusion, it is the author's conviction that the application of cytophotometric techniques for hormone microanalysis in physiologically active, morphologically heterogeneous, tissues such as the rat anterior pituitary contributes to a better understanding of endocrine responses during acclimation to a simulated high altitude environment.

SUMMARY

The response of the anterior pituitary gland during initial exposure and acclimation to a simulated high altitude of 18,000 feet was determined. Evidence of significant histophysiological changes was secured from either fixed whole pituitary glands or serial sections of the anterior pituitary. All tissues were obtained from male Sprague Dawley rats maintained either as controls at ambient pressure or as experimental groups continuously exposed to simulated altitude for one, two, seven, or 30 days. Tissue sections, chosen for definitive study, were stained differentially with alcian blue, P.A.S., and picric acid in accordance with the results of a preliminary study in which five differential anterior pituitary stains were compared.

Data obtained from differential cell counts of stained tissue sections showed that all chromophil cell types increased in relative number within 48 hours or less of the onset of altitude exposure. This was especially true of the acidophil cell series, while gonadotropic and thyrotropic basophil cells showed lesser increases. Chromophobe cells decreased in relative number in a manner corresponding to the increased chromophil population. Since no mitotic figures were observed, it was concluded that altitude-induced changes in relative anterior pituitary chromophil cell numbers did not represent true hyperplasia. Observed changes were judged to have resulted from accumulation of dye-binding hormone

materials within the cells of the pars distalis. Measurement of actual and relative pituitary weight changes during altitude acclimation provided further support for this conclusion.

Cytophotometric characterization of anterior pituitary chromophil cells of each type was shown to be possible using a Leitz microspectrophotometer. The similarities in absorption spectra obtained from the stained cytoplasm of individual cells of each type in both control and altitude-exposed rats demonstrated that hypoxia did not cause cytochemical alterations of any kind in the hormone products stored by respective chromophils.

Quantitative evaluation of the cytophotometric data indicated that the relative intensity of cytoplasmic staining increased in acidophil cells during altitude acclimation. Gonadotropic and thyrotropic basophils also displayed measurable increases in relative cytoplasmic staining intensity in all altitude-exposed animals. It was concluded that such increases in dye-binding capacity resulted from increased intracellular hormone storage.

Thus, all hormone-producing cell types within the male rat pars distalis display increased amounts of intracellular hormone material as a result of hypoxia exposure. Knowledge of this pituitary response furthers our understanding of the endocrine mechanisms by which the rat acclimates to conditions of simulated high altitude.

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APPENDIX A

I. Preparation of Reagents.

A. Schiff's reagent (leucofuchsin).

1. Distilled H_2O 480 ml
HCl, concentrated 20 ml
Sodium metabisulfite 9.5 g
Basic fuchsin (C.I. No. 42510) 5.0 g

2. Add the above, in order, to a flask; stopper tightly and shake at 2 minute intervals for a period of 2 hours.

3. Add 1 g of activated charcoal and shake continuously for 2 minutes.

4. Filter to remove the charcoal. The filtrate should be clear and colorless.

5. Store at 5 C until used.

B. Alcian blue.

1. Alcian blue (C.I. No. 74240) 0.1 g
Acetic acid, 3% 100 ml

2. Filter and add a crystal of thymol to preserve. The pH should be 2.5 to 3.0. Solution remains stable for 2 to 4 weeks.

3. Refilter if necessary before use.

C. Saturated picric acid.

1. Picric acid 1.18 g
Distilled H_2O 100 ml

D. Periodic acid.

1. Periodic acid 1.0 g
Distilled H_2O 100 ml

E. Bleaching solution

1. HCl, 1 N 60 ml
Sodium metabisulfite, 10% 60 ml
Distilled H_2O 1090 ml

2. Mix fresh before use.

II. Staining Procedure.

A. Alcian blue, P.A.S., picric acid stain for anterior pituitary.

1. Deparaffinize sections in xylene and hydrate through alcohols to water.
2. Stain in alcian blue for 30 minutes.
3. Wash in running tap water for 2 minutes.
4. Rinse in distilled water.
5. Oxidize in periodic acid for 5 minutes.
6. Rinse thoroughly in distilled water.
7. Place in Schiff's reagent for 20 minutes.
8. Rinse in 3 changes of bleaching solution for 2 minutes each.
9. Wash in running tap water for 10 minutes.
10. Stain in saturated picric acid for 1 minute.
11. Dip once in tap water.
12. Dip once in 95% alcohol.
13. Dehydrate quickly with 3 dips each in 2 changes of absolute alcohol.
14. Clear in xylene for 2 minutes and mount in Permount.

APPENDIX B

Summary Tables of the Data

Table 3. Cell count summary: Changes in relative abundance of acidophil, basophil, and chromophobe cells in the rat anterior pituitary in response to altitude exposure.[1]

Exposure (380 mm Hg)	n	Mean % Acidophils ± SE	P	Mean % all Basophils ± SE	P	Mean % Chromophobes ± SE	
Ambient pressure controls	5	28.2 ± 1.1		4.0 ± .4		67.9 ± 1.2	
1 day exposed	5	37.2 ± 3.2	.05	4.8 ± .3	0.2	58.0 ± 2.9	.02
2 day exposed	5	51.5 ± 1.9	.01	4.6 ± .6	0.4	43.9 ± 2.6	.01
7 day exposed	5	42.2 ± 2.2	.01	4.6 ± .9	0.6	53.2 ± 2.6	.01
30 day exposed	5	44.4 ± 3.3	.01	4.3 ± .4	0.5	51.3 ± 3.4	.01
1-30 day taken together	20	43.8 ± 1.3	.01	4.6 ± .2	0.02	51.6 ± 1.3	.01

[1]The symbol n refers to the number of rat pituitaries; P is the probability using Student's t-test. Total number of microscope fields counted = 2032; mean number of fields counted per animal = 81; mean number of total cells per field = 112; mean number of total cells per animal = 9072.

Table 4. Cell count summary: Changes in relative abundance of gonadotropic (type A) and thyrotropic (type B) basophil cells in the rat anterior pituitary in response to altitude exposure.[1]

Exposure (380 mm Hg)	n	Mean % Gonadotropic Basophils ± SE	P	Mean % Thyrotropic Basophils ± SE	P
Ambient pressure controls	5	3.2 ± .2		0.8 ± .4	
1 day exposed	5	3.6 ± .4	0.4	1.2 ± .3	0.2
2 day exposed	5	3.1 ± .7	0.8	1.5 ± .3	0.1
7 day exposed	5	3.7 ± .5	0.4	0.9 ±	0.8
1-30 day exposed	5	3.4 ± .3	0.4	0.9 ± .1	0.6
1-20 day taken together	20	3.4 ± .1	0.3	1.1 ± .1	0.2

[1]The symbol n refers to the number of rat pituitaries; P is the probability using Student's t-test. (See Table 3)

Table 5. Absorption data for Corning CS1-60 didymium glass filter: Effect of monochromator exit slit width upon spectral resolution.

Wavelength in mμ	% Absorption exit slit = 0.8 mm	% Absorption exit slit = 0.6 mm	% Absorption exit slit = 0.3 mm	Mean % Absorption
400	24	30	27	27
420	18	13	16	16
440	52	53	53	53
460	47	54	54	52
480	42	46	50	46
500	42	43	39	41
520	69	70	74	71
540	30	25	20	25
560	63	65	71	66
580	95	97	99	97
600	67	61	52	60
620	24	18	13	18
640	16	6	11	11
660	18	13	13	15
680	19	17	25	20
700	23	34	0	19

Table 6. Spectral characterization of acidophil cells from ambient pressure control and altitude-exposed rats.

Wave length in mμ	Mean % Abs. controls n = 15	Mean % Abs. 1 day exposed n = 15	Mean % Abs. 2 day exposed n = 15	Mean % Abs. 7 day exposed n = 15	Mean % Abs. 30 day exposed n = 15	Mean % Abs. all animals n = 75	Range Mean % Abs. all animals
400[1]	80.0 ± 1.5	84.3 ± 1.5	83.1 ± 1.9	82.3 ± 1.5	84.3 ± 1.4	83.0 ± 1.5	71-92
420	78.7	81.1	80.0	79.3	82.0	80.3	67-90
440	60.6	63.3	61.9	61.8	65.2	62.5	46-76
460	28.3	31.3	30.9	31.4	32.6	31.1	19-49
480[1]	17.1 ± 1.1	19.8 ± 1.5	18.1 ± 1.9	21.3 ± 2.9	19.3 ± 2.1	19.1 ± 1.9	7-43
500	19.9	23.9	20.5	25.9	25.2	23.1	15-48
520	25.8	30.4	24.8	31.5	32.1	28.8	16-50
540	29.9	36.7	31.0	37.7	38.5	34.8	22-55
560[1]	33.7 ± 1.8	41.1 ± 2.1	36.9 ± 2.3	42.9 ± 1.8	43.3 ± 1.6	39.6 ± 1.9	27-56
580	33.4	39.7	39.2	44.0	44.7	40.1	28-58
600	26.7	36.2	35.8	38.7	40.5	35.4	22-58
620	22.6	32.8	33.9	35.6	36.7	32.3	12-55
640	18.1	26.9	28.9	30.1	30.8	27.0	7-45
660[1]	15.2 ± 1.8	22.0 ± 1.5	25.7 ± 1.6	23.5 ± 1.6	25.6 ± 1.4	22.4 ± 1.6	7-35
680	16.3	22.1	25.9	24.0	27.1	23.1	10-35
700	20.3	23.7	24.9	24.5	25.5	23.8	9-46

Symbol n equals number of cells analyzed with the cytophotometer.

[1]Standard Error of Mean % Abs. calculated for these wavelengths.

Table 7. Spectral characterization of gonadotropic (type A) basophil cells from ambient pressure control and altitude exposed rats.

Wave-length in mμ	Mean % Abs. controls n = 15	Mean % Abs. 1 day exposed n = 15	Mean % Abs. 2 day exposed n = 15	Mean % Abs. 7 day exposed n = 14	Mean % Abs. 30 day exposed n = 15	Mean % Abs. all animals n = 73	Range Mean % Abs. all animals
400[1]	60.6 ± 2.4	68.8 ± 1.7	66.9 ± 1.9	68.7 ± 1.5	74.9 ± 2.6	68.1 ± 2.0	50-92
420	55.6	72.9	60.1	60.7	69.1	61.8	48-91
440	41.6	46.7	44.7	45.3	53.1	46.3	32-78
460	24.7	29.9	28.9	28.8	33.1	29.1	19-46
480[1]	22.9 ± 1.7	30.7 ± 1.6	31.2 ± 1.9	32.0 ± 1.7	31.5 ± 1.9	29.8 ± 1.8	15-42
500	32.6	44.0	43.9	46.1	45.8	42.6	22-57
520	42.8	55.7	53.3	56.6	57.7	53.3	32-67
540	47.9	61.3	59.0	63.4	64.3	59.3	40-74
560[1]	49.7 ± 1.5	64.3 ± 1.8	63.2 ± 2.8	66.8 ± 1.7	68.5 ± 2.4	62.6 ± 2.0	44-79
580	47.0	62.7	62.1	64.5	67.8	61.1	40-80
600	37.7	55.1	57.1	57.6	60.9	53.8	30-75
620	29.4	44.1	45.4	44.1	49.0	42.6	18-64
640	20.3	36.7	37.0	35.8	41.7	34.5	6-58
660[1]	19.8 ± 1.7	34.7 ± 1.8	33.5 ± 2.4	32.5 ± 1.9	38.5 ± 2.2	32.0 ± 2.0	12-53
680	24.9	37.7	35.2	32.1	39.0	33.9	12-50
700	29.3	42.7	38.1	40.1	47.7	39.7	13-60

Symbol n equals number of cells analyzed with the cytophotometer.

[1]Standard Error of Mean % Abs. calculated for these wavelengths.

Table 8. Spectral characterization of thyrotropic (type B) basophil cells from ambient pressure control and altitude-exposed rats.

Wave-length in mμ	Mean % Abs. controls n = 15	Mean % Abs. 1 day exposed n = 14	Mean % Abs. 2 day exposed n = 15	Mean % Abs. 7 day exposed n = 14	Mean % Abs. 30 day exposed n = 14	Mean % Abs. all animals n = 72	Range Mean % Abs. all animals
400[1]	56.3 ± 2.8	58.6 ± 3.2	59.9 ± 2.1	59.2 ± 2.4	63.2 ± 2.4	58.7 ± 2.4	41-77
420	51.1	52.6	47.9	51.5	56.5	52.1	35-70
440	36.1	36.2	32.9	35.4	40.5	36.5	18-53
460	19.0	20.9	19.6	18.5	22.6	20.5	6-47
480[1]	17.8 ± 2.9	21.4 ± 2.3	18.5 ± 1.4	17.9 ± 1.8	19.9 ± 1.8	19.5 ± 1.8	8-47
500	25.2	30.9	27.8	24.6	26.7	27.3	15-50
520	30.7	37.1	34.1	32.1	37.9	34.6	17-56
540	33.7	41.4	36.5	35.4	42.3	37.9	17-56
560[1]	35.4 ± 2.5	43.9 ± 2.6	39.3 ± 2.0	39.1 ± 2.0	44.1 ± 3.0	40.5 ± 2.5	22-61
580	31.1	40.9	35.1	36.8	43.3	37.5	20-64
600	22.9	33.9	28.8	30.5	37.1	30.7	9-54
620	18.2	26.6	23.5	24.5	29.3	24.6	10-43
640	14.9	20.9	19.7	19.9	21.7	19.8	6-44
660[1]	14.9 ± 1.2	18.4 ± 2.4	16.2 ± 1.6	15.4 ± 1.5	19.4 ± 0.9	17.2 ± 1.2	6-44
680	19.1	18.3	17.7	15.1	20.6	18.5	10-40
700	28.4	22.3	20.7	17.2	23.9	22.9	9-40

Symbol n equals number of cells analyzed with the cytophotometer.

[1]Standard Error of Mean % Abs. calculated for these wavelengths.

Table 9. Spectral quantification of intracellular hormone in chromophil cell types of ambient pressure control and altitude-exposed rats. (Height of major absorption peaks is recorded in optical density units.)

Exposure Duration (380 mm Hg)	n	Acidophil Cells Mean O.D. ± SE at 500 mμ	n	Gonadotropic Basophil Cells Mean O.D. ± SE at 560 mμ	n	Thyrotropic Basophil Cells Mean O.D. ± SE at 560 mμ
Ambient pressure controls	15	.721 ± .004	14	.301 ± .009	15	.187 ± .013
1 day exposed	15	.796 ± .009	15	.444 ± .009	14	.252 ± .013
2 day exposed	15	.770 ± .009	15	.432 ± .013	15	.215 ± .009
7 day exposed	15	.745 ± .009	14	.482 ± .009	14	.215 ± .009
30 day exposed	15	.824 ± .004	15	.509 ± .009	14	.252 ± .013

n = number of cells spectrally analyzed.