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Mid-Term Report

for

NASA Contract NAS 9-15566

Purification and Cultivation of Human

Pituitary Growth Hormone Secreting Cells

(NASA-CF-151853) PURIFICATION AND  
CULTIVATION OF HUMAN PITUITARY GROWTH  
HORMONE SECRETING CELLS Mid-term Report  
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by

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November 1, 1978

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ADDENDUM

## I. General Introduction

Our efforts are being directed toward maintenance of actively secreting human pituitary growth hormone cells (somatotrophs) in vitro. The production of human growth hormone (hGH) by this means would be of benefit for the treatment of certain human hypopituitary diseases such as dwarfism. Since the chemical nature of the factor(s) responsible for stimulating the release of hGH from the somatotroph is totally unknown, one of our primary approaches has (and will continue to be) testing of agents which may be expected to increase hGH release. This mid term report briefly summarizes our progress towards this goal.

## II. Tissue Procurement

Shortly after initiation of this contract, we began setting up procedures for the procurement of human pituitary tissue. The following pathologists and neurosurgeons are collaborating on this project:

Dr. Andrew Dekker, University of Pittsburgh, Pittsburgh, PA

Dr. Olof Pearson, Case Western Reserve, Cleveland, Ohio

Dr. Robert Page, Hershey Medical Center, Hershey, PA

Dr. Thomas Pretlow, University of Alabama, Birmingham, AL

Dr. Bruce Northrup, Thomas Jefferson Medical School, Philadelphia, PA

In all cases, these individuals mince the tissue into  $\sim 1 \text{ mm}^3$  fragments and send them to Penn State in bottles containing 50 ml of sterile medium 199 containing 5% horse serum and pen-strep (100 $\mu$ /ml). These bottles, which are shipped at ambient temperature, usually arrive in our laboratory within 24 hours after removal of the gland. Our samples from Pittsburgh usually arrive within 18 hrs. Pertinent tissue information is provided in Table I. Note that in 4 cases thus far, samples of tissue were obtained at surgery (biopsy) from patients with either breast cancer or pituitary tumors.

TABLE I

| Sample Number<br>and Sex of Donor | Source | Age of<br>Donor | AUTOPSY SPECIMEN    |  | BIOPSY SPECIMEN         |
|-----------------------------------|--------|-----------------|---------------------|--|-------------------------|
|                                   |        |                 | Time After<br>Death | Cause                                    | Cause                   |
| 1 (M)                             | ALA    | 70              | 4 hrs.              | Heart Failure                            | ----                    |
| 2 (F)                             | CLE    | 55              | ----                | ----                                     | Metastatic CA of Breast |
| 3 (F)                             | CLE    | 75              | ----                | ----                                     | Metastatic CA of Breast |
| 4 (F)                             | HER    | 75              | ----                | ----                                     | Acromegalic             |
| 5 (F)                             | PHL    | 67              | ----                | ----                                     | Pituitary Tumor         |
| 6 (F)                             | PIT    | 69              | 7 hrs.              | Metastatic Adenocarcinoma                | ----                    |
| 7 (M)                             | PIT    | 78              | 6 hrs.              | Emphysema, Pneumonia                     | ----                    |
| 8 (M)                             | PIT    | 61              | 6 hrs.              | Congestive Heart Failure,<br>GI Bleeding | ----                    |
| 9 (M)                             | PIT    | 64              | 3 hrs.              | Malignant Neoplastic<br>Bronchus         | ----                    |
| 10 (M)                            | PIT    | 58              | 5 hrs.              | Metastatic Squamous Cell<br>Cancer       | ----                    |
| 11 (M)                            | PIT    | 64              | 2 hrs.              | Adenocarcinoma of<br>the stomach         | ----                    |
| 12 (M)                            | PIT    | 64              | 6 hrs.              | Ruptured Abdominal Aneurism              | ----                    |
| 13 (M)                            | PIT    | 58              | 6 hrs.              | Lung Cancer                              | ----                    |

## II. A. Dissociation Procedures

The human pituitary is composed of epithelial cells supported by a significant framework of fibrous connective tissue. It is therefore not surprising that an enzymatic dissociation utilizing collagenase type I will yield more cells than either collagenase type III (which presumably contains more lipase) or trypsin alone (Table II). The chemicals used in Method I (see Table II) were suggested to us by Dr. Gary Mulder of New York University Medical School (personal communication). Thus far, our experience indicates it is the method of choice. Cell viability is usually >85%. Note that cell yield from autopsy material can be quite comparable to that obtained from "fresh" material (cf sample 12 vs 4, Table II). Dr. Mulder informs me that his cell yields from autopsy material are also  $\sim 2 \times 10^6$  cells/gland.

## II. B. Light Microscopic Histology

Herlants tetrachrome stain is especially useful for the unequivocal differentiation and identification of pituitary somatotrophs. Yellow-stained somatotrophs prepared from "fresh" pituitaries (breast cancer patients - Fig. 1a, b, c) or autopsy pituitary tissue (Fig. 1e, f) were commonly encountered. The fact that such cells assume a yellow stain can be taken as positive evidence that they do indeed retain their intracellular stores of GH upon dissociation. Examples of blue staining cells (basophils-TSH, FSH and LH cells) are also seen in these preparations. Using such morphological criteria, we conclude that cells from autopsy material are not different from fresh material. This in turn suggests the feasibility of using autopsy material for experimental work.

The cells in Fig. 1d were prepared from an acromegalic patient undergoing hypophysectomy. Even though this patient's serum GH levels were elevated, the cells did not take on a yellow stain in their cytoplasm. Instead, they have the typical appearance of tumorous cells. It is commonly believed that turnover of GH is high in such cells, i.e., once made, the hormone molecules are rapidly released.

TABLE II

| Sample | Dissociation Method | Cell Yield  | Autopsy (A) or Biopsy (B) |
|--------|---------------------|---|---------------------------|
| 2      | 3<br>1              | $1.7 \times 10^6 / 0.5$ PIT<br>$2.7 \times 10^6 / 0.5$ PIT  | B                         |
| 3      | 1                   | $1.5 \times 10^6$   | B                         |
| 4      | 1                   | $3.1 \times 10^6$   | B                         |
| 5      | 1                   | $0.4 \times 10^6$   | B                         |
| 6      | 1                   | NOT COUNTED   | A                         |
| 7      | 2<br>1              | $0.04 \times 10^6 / 0.5$ PIT<br>$0.3 \times 10^6 / 0.5$ PIT | A                         |
| 8      | 1<br>2              | NOT COUNTED   | A                         |
| 11     | 1                   | $1.1 \times 10^6$   | A                         |
| 12     | 1                   | $2.5 \times 10^6$   | A                         |
| 13     | 1                   | $0.3 \times 10^6$   | A                         |

KEY

Method 1: Medium 199 + 0.5% BSA + 0.3% collagenase I

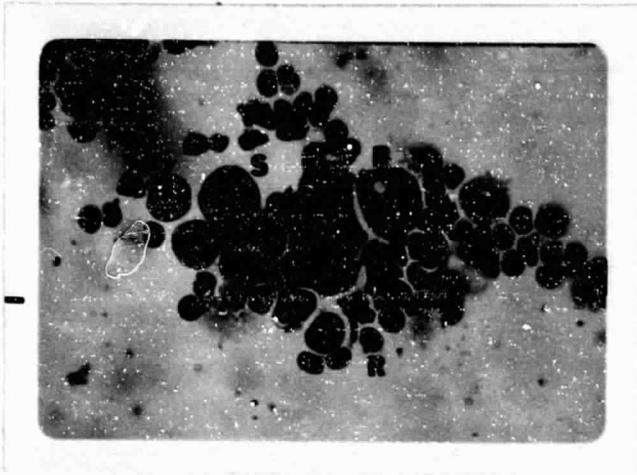
Method 2: MEM + 0.1% BSA + 0.1% trypsin (1:250)

Method 3: Hanks + 0.1% BSA + 0.3% collagenase III followed by 199 + 0.1% BSA + 0.25% VIOKASE

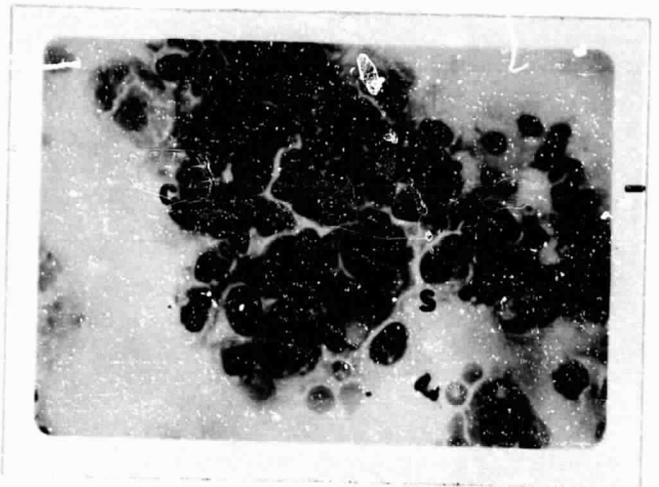
Fig. 1. Light microscopic photomicrographs of human pituitary cells prepared by enzymatic dissociation (Table II) and stained with Herlants tetrachrome stain. The cells with yellow staining cytoplasm are somatotrophs (S) whereas those with blue cytoplasm are basophils (B) - i.e., either TSH, FSH or LH producing cells. RBC's (R), chromophobes (probably prolactin cells) (C), and fibrous connective tissue (F) are also identified.

- a) Sample #3 - breast cancer pituitary
- b) Sample #2 - breast cancer pituitary
- c) Sample #2 - breast cancer pituitary
- d) Sample #4 - acromegalic pituitary
- e) Sample #7 - autopsy pituitary
- f) Sample #7 - autopsy pituitary

(See Table I for tissue information).

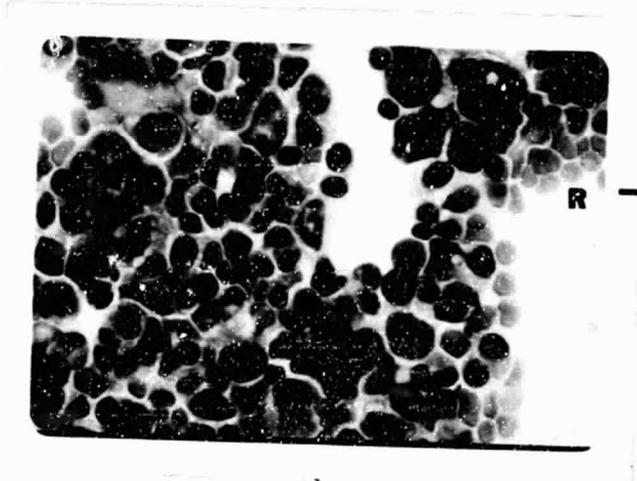


a

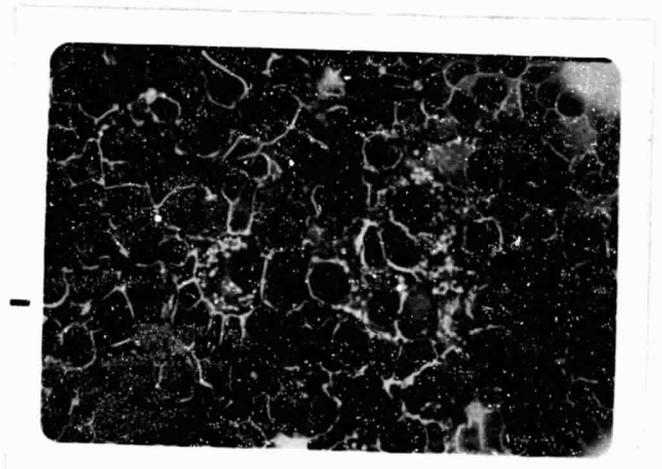


b

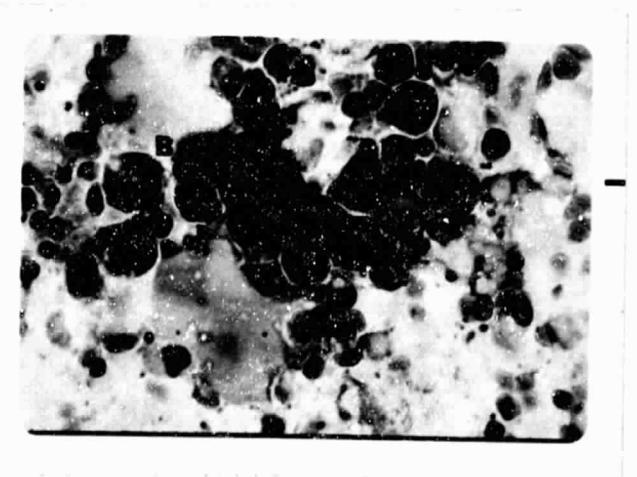
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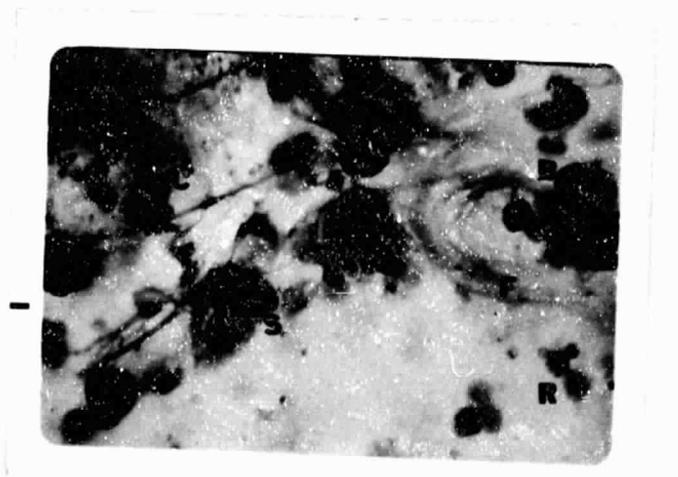
c



d



e



f

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### III. Biogel Column Perfusion

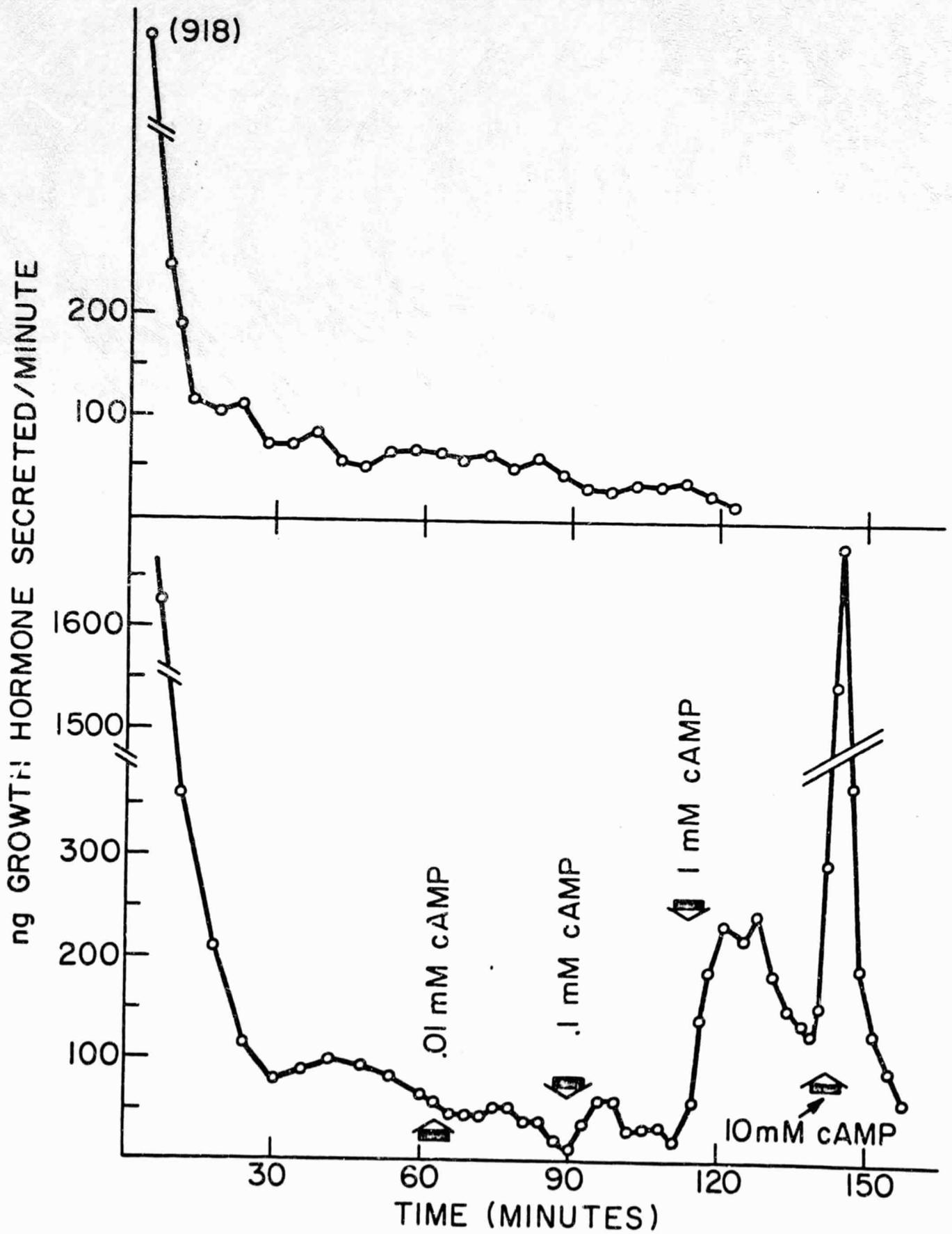
Previous experience from our laboratory (Snyder and Hymer, Endo., Sept. '77; Snyder, Hymer and Wilfinger, Cell and Tissue Research, Aug., 1978; Wilfinger et al., Endo., in press), as well as from those of others, clearly shows that it is extremely difficult to maintain actively secreting somatotrophs in conventional monolayer for any significant period of time. This difficulty is probably due 1) to lack of knowledge as to the chemical nature of native growth hormone releasing factor and 2) to rapid overgrowth of epithelial cells by fibroblasts. In 1974 a simple, yet powerful, technique was described by Lowry and his coworkers to study the dynamics of hormone release from pituitary cells immobilized in columns of Biogel. This technique has the major advantage of removing potentially harmful metabolites from the immediate vicinity of the cells by constant perfusion of the system. In the next section I will briefly relate our experiences with rat pituitary cells in Biogel so that the reader can get a feeling for the usefulness of this technique. After that, our preliminary experiences with human cells in the column will be described.

#### III. A. Rats

In our usual experiment,  $5 \times 10^6$  pituitary cells are packed in a 2 ml syringe with 0.15 gms. of preswollen Biogel P-2 (column height = 0.8 ml). The bottom of the column is constructed with a "sandwich" of nytex (20 $\mu$ ) and millipore (3 $\mu$ ) filters to prevent cells from escaping the column. Buffer is pumped into the top of the column (flow rate = 0.5 ml/min) by means of a Manosette Cassette Pump and column eluant is collected into tubes with a fraction collector. Both the buffer vessel and column are immersed in a 37° water bath. Lines connecting with the main inlet line by 3-way valves permit one to accurately "pulse" the cells with a variety of secretagogues at any desired interval.

Shown in Figure 2 (top) is "basal" GH secretion. Note that 30-60 minutes after starting the experiment a relatively constant hormone release rate is

Fig. 2



achieved. The response of the cells to graded doses of dibutyryl cyclic AMP (0.01-10.0 mM) is also shown in Fig. 2 (bottom). A 2-minute pulse with this cyclic nucleotide will stimulate GH release in dose-related fashion at concentrations above 0.1 mM. This is a highly repeatable response in the rat system. In other experiments (not shown) the response can be potentiated with theophylline, a phosphodiesterase inhibitor. In these experiments the column buffer was Medium 199 containing 0.1% BSA.

The versatility of the Biogel column technique is exemplified by the data shown in Fig. 3. In this experiment 3 columns, each containing  $5 \times 10^6$  cells, were run concurrently. The response to repeated 2' pulses of 1 mM cAMP is clearly evident in Fig. 3A. Inclusion of somatostatin (SIF) in the column buffer dampens basal release and prevents cAMP stimulated release (Fig. 3B). Perfusion of the cells with SIF immediately after cAMP will, for a time, dampen the secretory response. Eventually, however, large "breakthrough" peaks are routinely encountered (Fig. 3C).

Our experiences with prostaglandins (E series, E<sub>1</sub> and E<sub>2</sub>) are typified by the data in Fig. 4. A sharp rise ( $\sim 420$  ng to  $\sim 1650$  ng) in GH release occurs within 1 minute after pulsing with  $10^{-9}$  M PGE<sub>2</sub>. A less dramatic response was obtained with  $10^{-8}$  M PGE<sub>2</sub> (Fig. 4, top). We have also noted an interesting difference between the responsiveness of lightly granulated (Type I) vs heavily granulated (Type II) somatotrophs to  $5 \times 10^{-10}$  M PGE<sub>1</sub> (Fig. 4, bottom). The two types of somatotrophs were separated on the basis of density differences, i.e., density gradient centrifugation through heavy BSA.

In summary, these data collected on rat pituitary somatotrophs have prompted us to begin comparable experiments with human pituitary tissue.

### III. B. Humans

1. hGH RIA. To do these experiments it was first necessary to obtain a hGH RIA kit and establish the assay in our laboratory.

Fig. 3

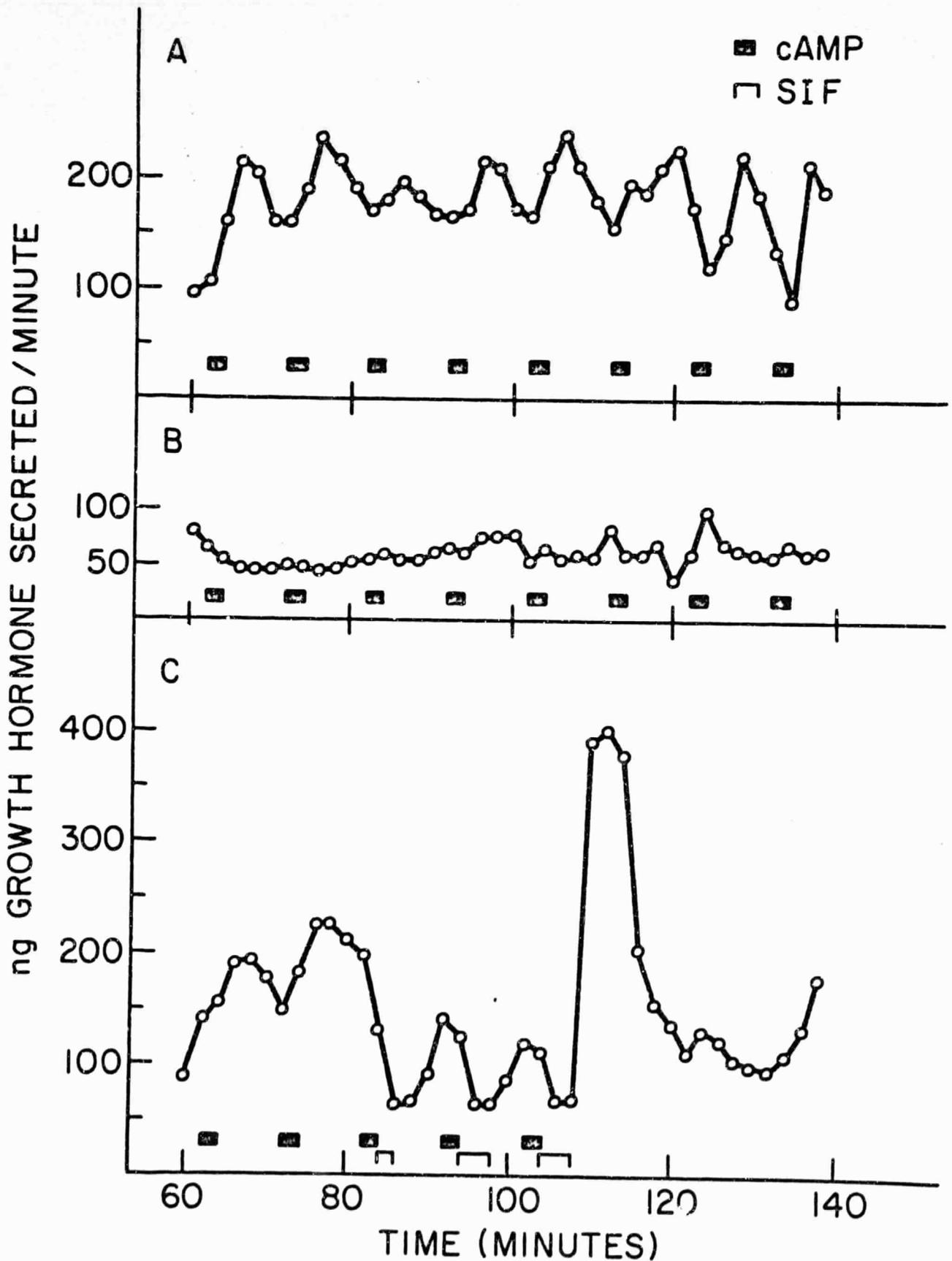
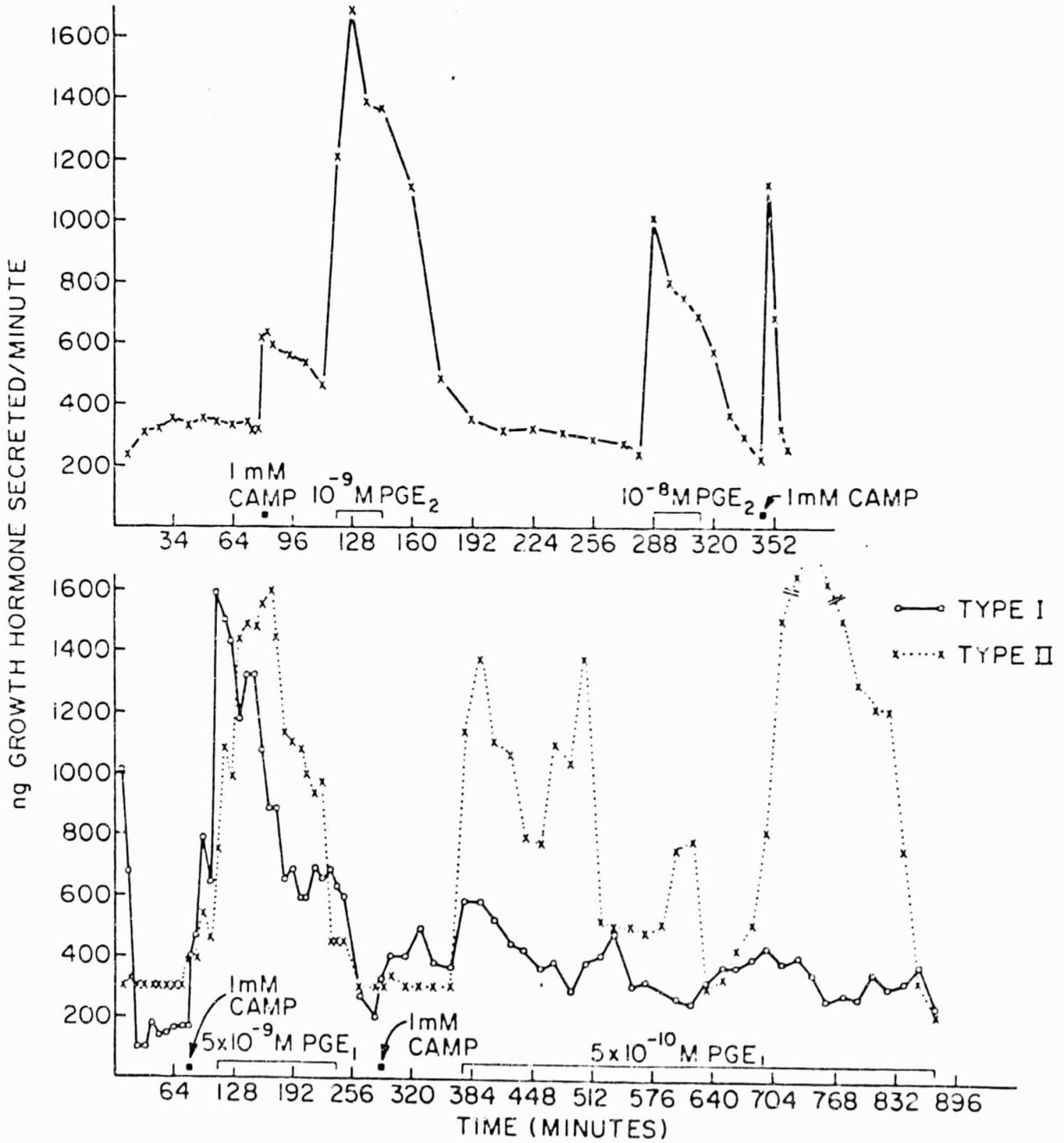
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Fig. 4



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The assay employs the immunochemical hGH (Batch #HS2243E) and guinea pig antiserum to hGH provided by NIAMDD and a sheep anti-guinea pig gamma globulin serum. Factors affecting the iodination of hGH with  $^{125}\text{I}$  and assay variables such as antibody and  $^{125}\text{I}$  concentrations have been critically examined (Figs. 5A and 5b). The lower limit of sensitivity of the assay is 0.025 ng, allowing detection of as little as 0.25 ng of hGH/ml of sample.

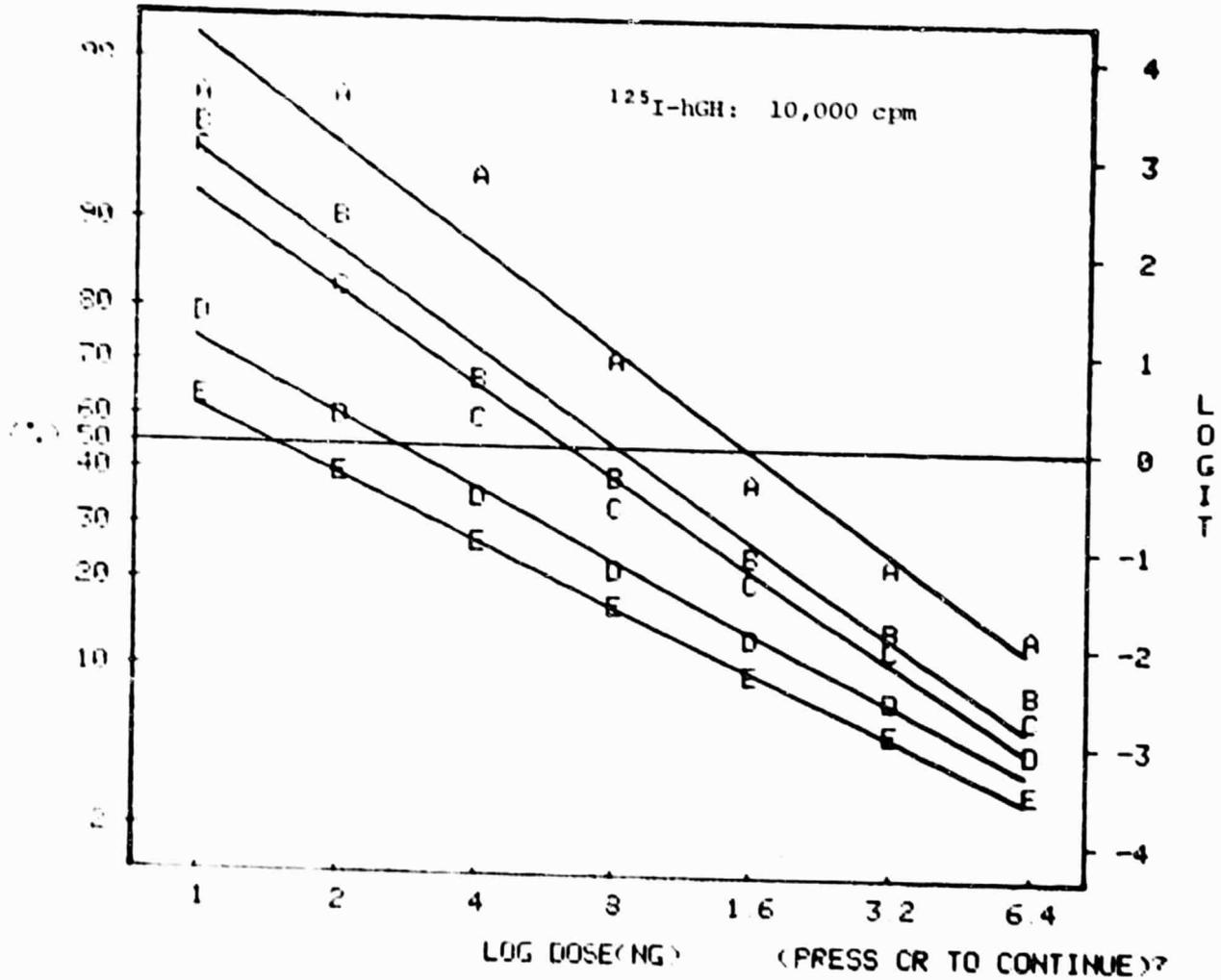
2. "Pieces". While we were gaining experience with the enzymatic dissociation of human pituitary tissue (Table II) we carried out 2 separate experiments in which minced pituitary pieces ( $1\text{ mm}^3$ ) were placed on top of the biogel column. In both of these experiments 2 columns running concurrently were used, each receiving the equivalent (in wet weight) of half of the available tissue (essentially 1/2 of a gland. Pituitary glands from autopsy material were used in both experiments (cross reference Fig. 6 with Table I for detailed information). In both experiments, one column buffer was medium 199 + 0.1% BSA + secretagogue (cAMP or  $\text{PGE}_1$ ) while the other column buffer was identical except it also contained  $10^{-9}$  M hydrocortisone (HC). This was included because we had shown in earlier studies that this steroid promoted synthesis, but not release, of GH in rat somatotrophs.

From the results in Fig. 6 the following conclusions are drawn:

- a) Over the 5 1/2 hour period, the quantity of GH ranged 300-800 ng/ml (note ordinate scale change between Fig. 6 top and bottom).
- b) After an initial period of rapid decline in GH release (also seen in the rat system, e.g., Fig. 2, 3, 4), "basal" levels remained fairly constant after  $\sim 1$  hr.
- c) Addition of hydrocortisone to the medium had no effect on GH release over this relatively short time period. (Longer time periods are currently being tested).
- d) cAMP (30' pulses) did not stimulate GH release as it does in the rat. In fact, at the high dose it tended to suppress release.

Fig. 5A

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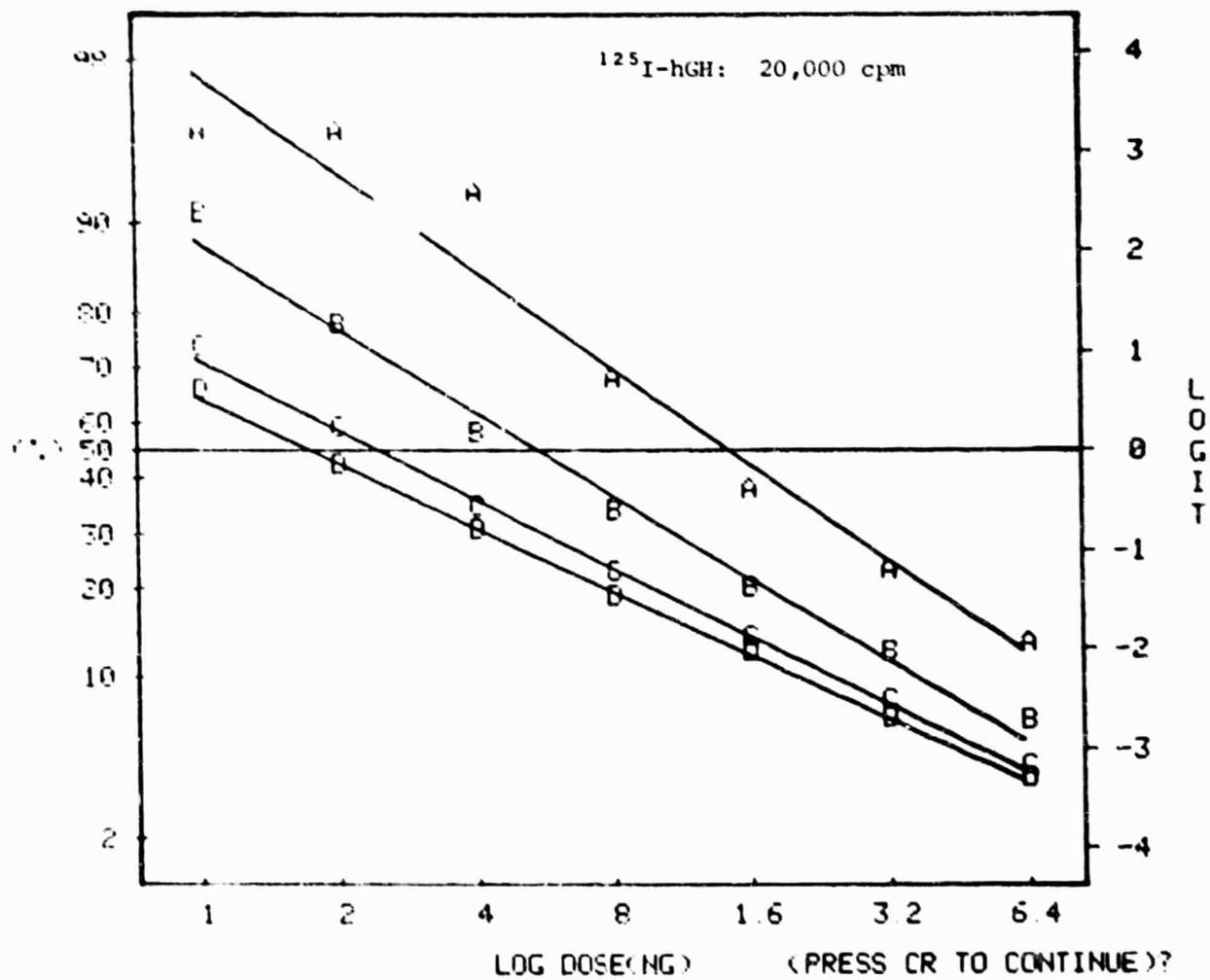


Standard Curve

Final Antibody Dilution

|   |             |
|---|-------------|
| A | 1/200,000   |
| B | 1/400,000   |
| C | 1/500,000   |
| D | 1/1,000,000 |
| E | 1/2,000,000 |

Fig. 5B



Standard Curve

A  
B  
C  
D

Final Antibody Dilution

1/200,000  
1/500,000  
1/1,000,000  
1/2,000,000

e) There tended to be a "rebound recovery" in GH release after the pulse of 10 mM cAMP.

f) PGE<sub>1</sub> had little effect on GH release under these particular experimental conditions.

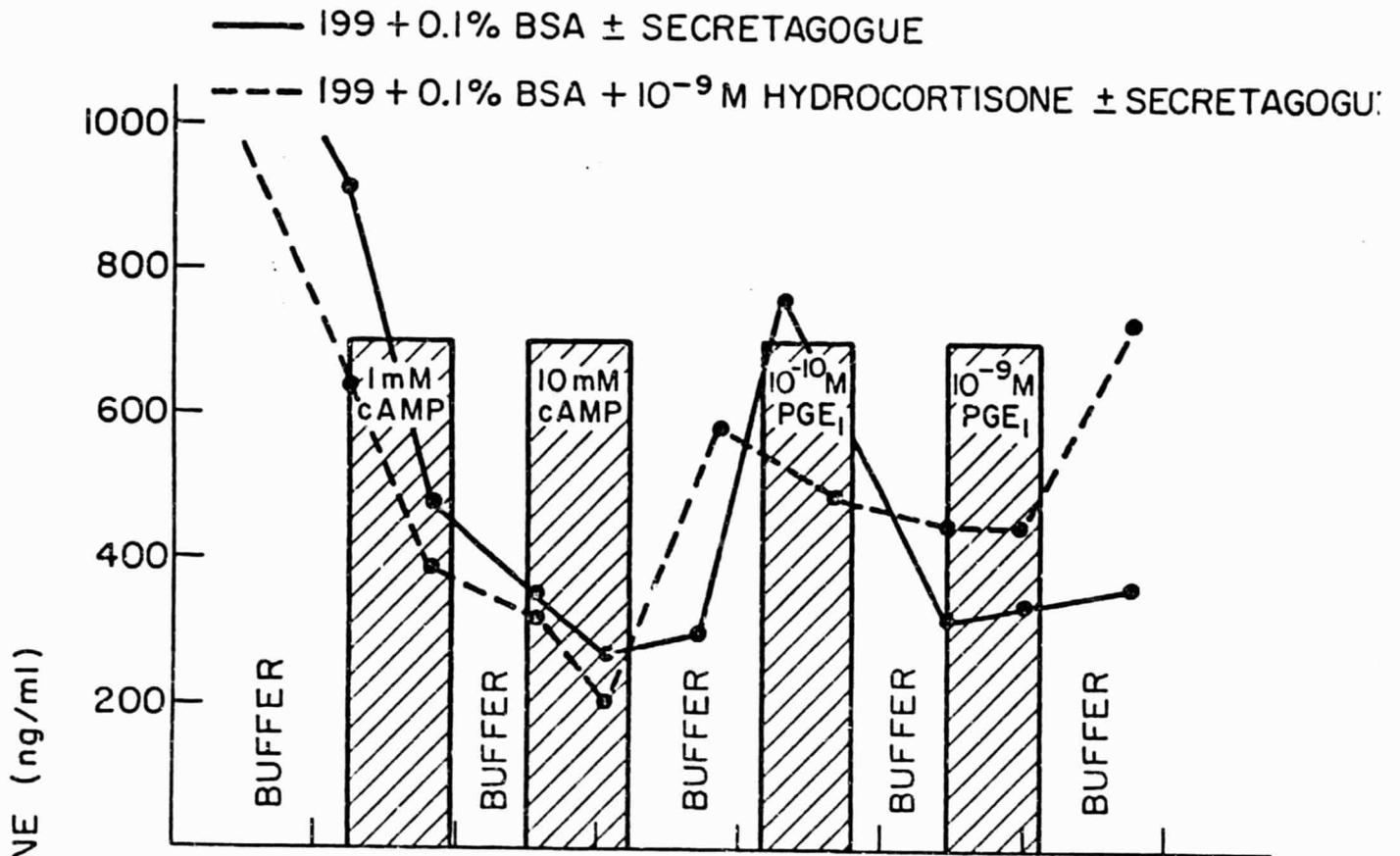
3. Cells - Autopsy Material. Our preliminary experiences with the GH secretory performance of dispersed cells prepared from autopsy material (see Table II for methodology) in the Biogel column system is depicted in Figs. 7 and 8. Note that the initial high GH levels in the early fractions fall precipitously to a relatively constant basal rate  $\sim$  1 hr. after the start of the experiment. This fall can probably not be attributed to the dissociation procedure since it also occurred when "pieces" were used (cf Fig. 6). It would appear that the level of basal secretion is related to the amount of tissue in the column. Thus, it was  $\sim$  25 ng/ml when  $1.1 \times 10^6$  cells were used (Fig. 7);  $\sim$  50 ng/ml when  $2.2 \times 10^6$  cells were used (Fig. 8) and  $\sim$  600 ng/ml when  $\sim$  1/2 of a pituitary gland was used (Fig. 6).

It is obvious from Fig. 7 and Fig. 8 that the combination of  $10^{-9}$  M PGE<sub>1</sub> and  $10^{-9}$  M hydrocortisone does not augment release of hGH over the time periods (up to 6 hrs.) of these experiments. Also note that inclusion of serum in the column buffer does not affect the basic release pattern. At the time of writing this report we are conducting a series of experiments to examine the effects of PGE<sub>1</sub> and HC on GH release from somatotrophs maintained in Biogel for much longer periods (> 24-36 hrs.).

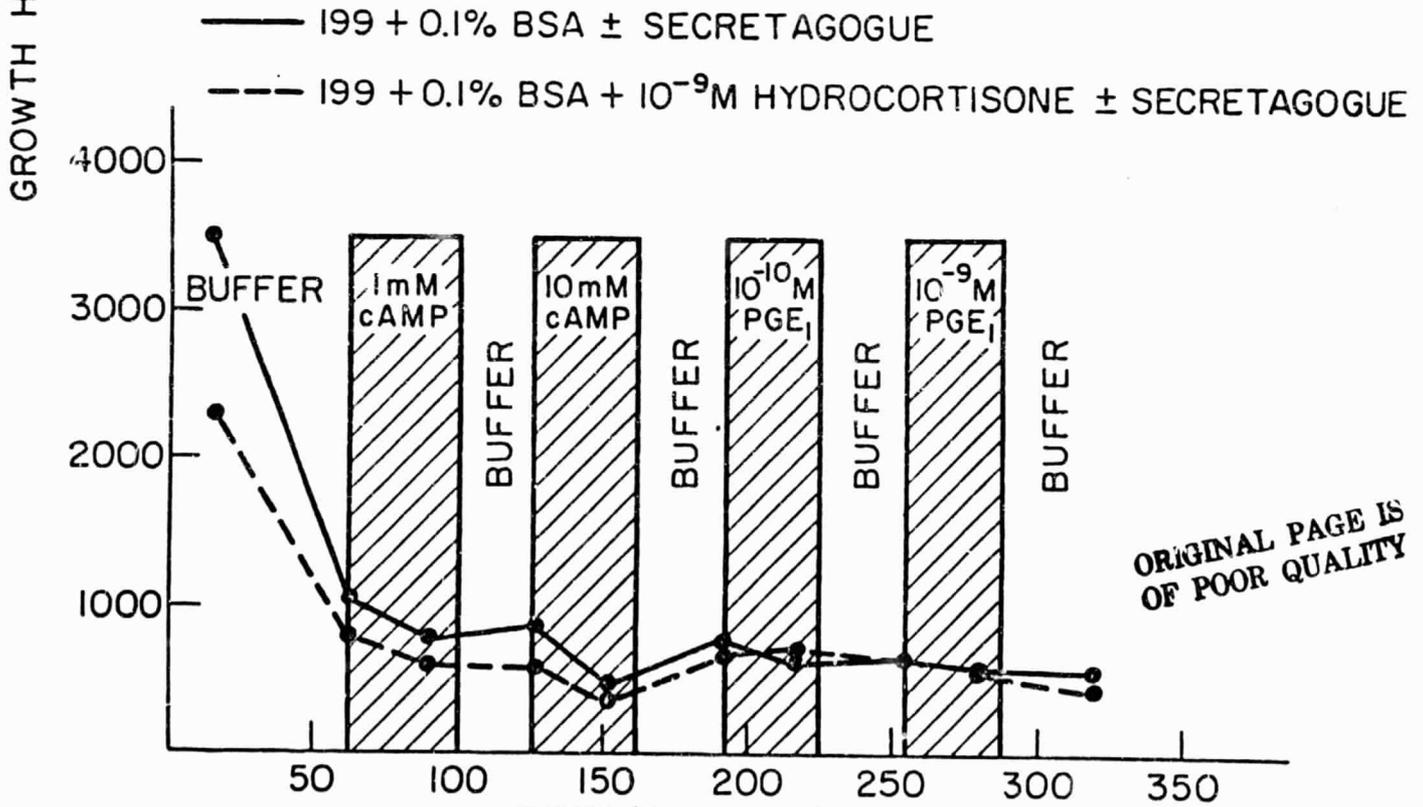
4. Cells - Biopsy Material. The dynamics of GH release from dispersed cells prepared from fresh pituitary tissue (i.e., surgical specimens) are shown in Figs. 9, 10 and 11. In two cases the basal levels are surprisingly low ( $\sim$  5 ng/ml). We have no explanation for this result at the present time.

The basal release levels from an approximately equivalent number of acromegalic cells ( $3.8 \times 10^6$  cells) was considerably higher ( $\sim$  300 ng/ml, Fig. 11), a

SAMPLE 1 : PIECES



SAMPLE 10 : PIECES



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Fig. 7

SAMPLE II: CELLS ( $1.1 \times 10^6$ ) - AUTOPSY  
BUFFER: 199 + 0.1% BSA  $\pm$  PGE<sub>1</sub> + HC

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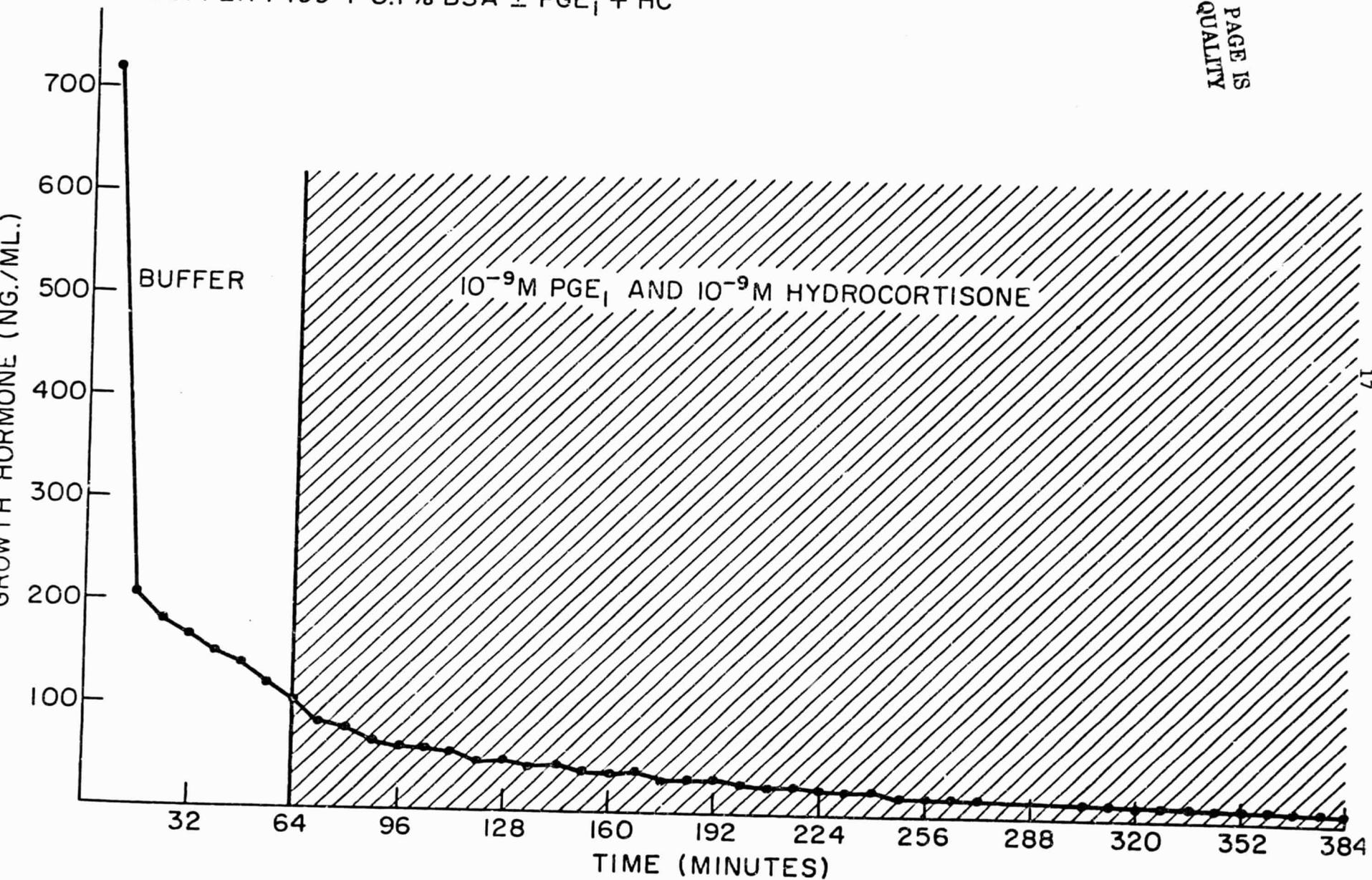


Fig. 8

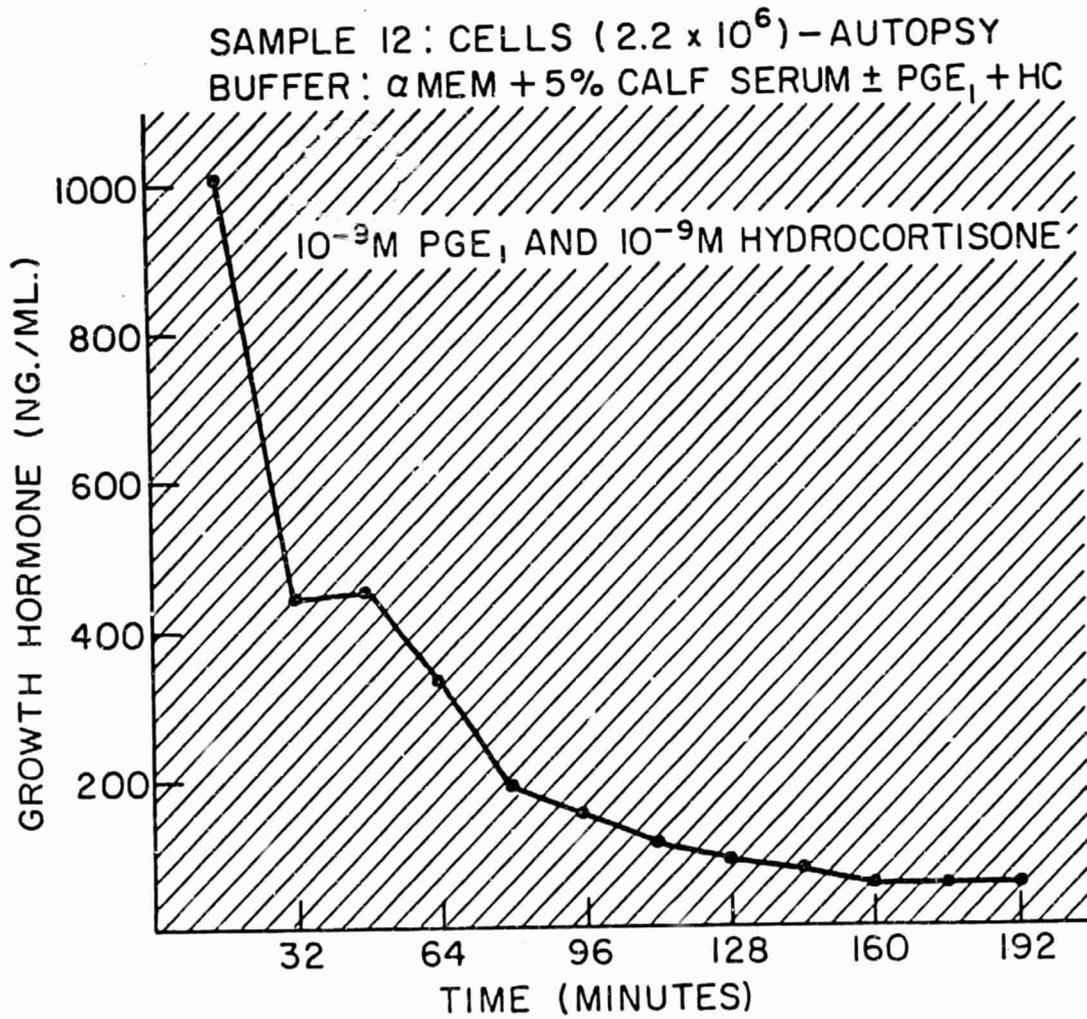


Fig. 9

SAMPLE 3 : CELLS ( $1.5 \times 10^6$ ) - BIOPSY  
BUFFER : 199 + 0.1% BSA  $\pm$  PGE<sub>1</sub> + HC

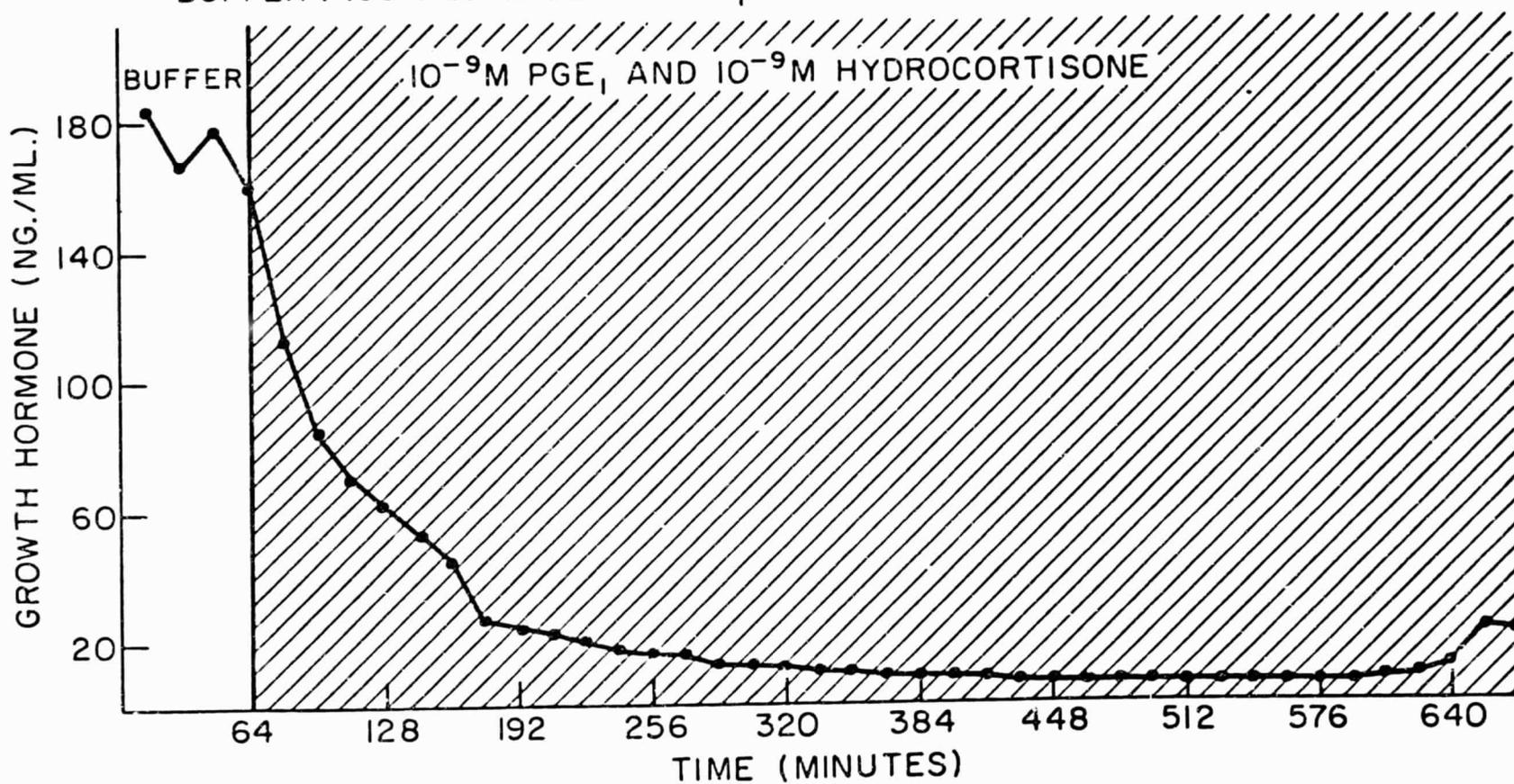
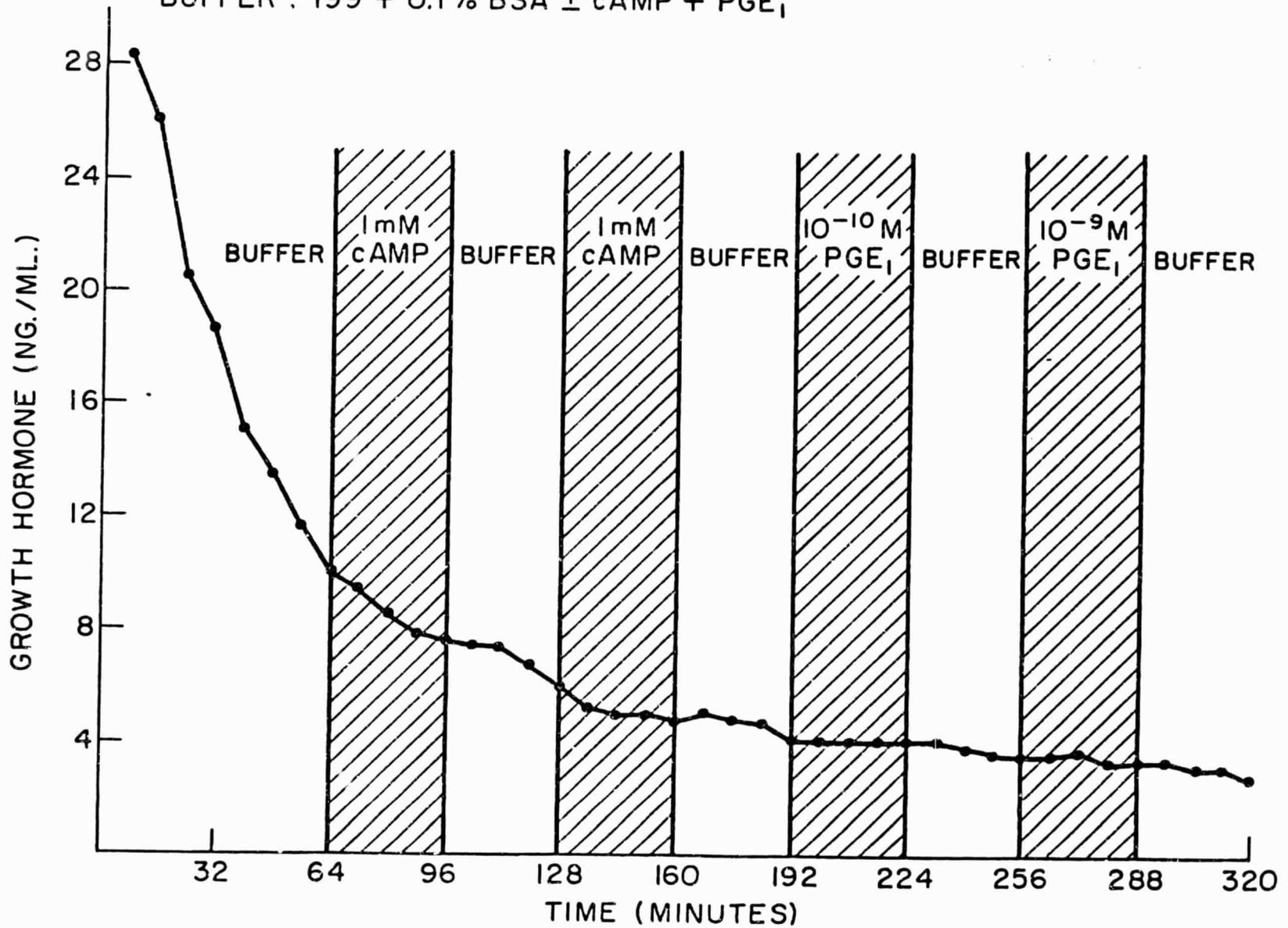


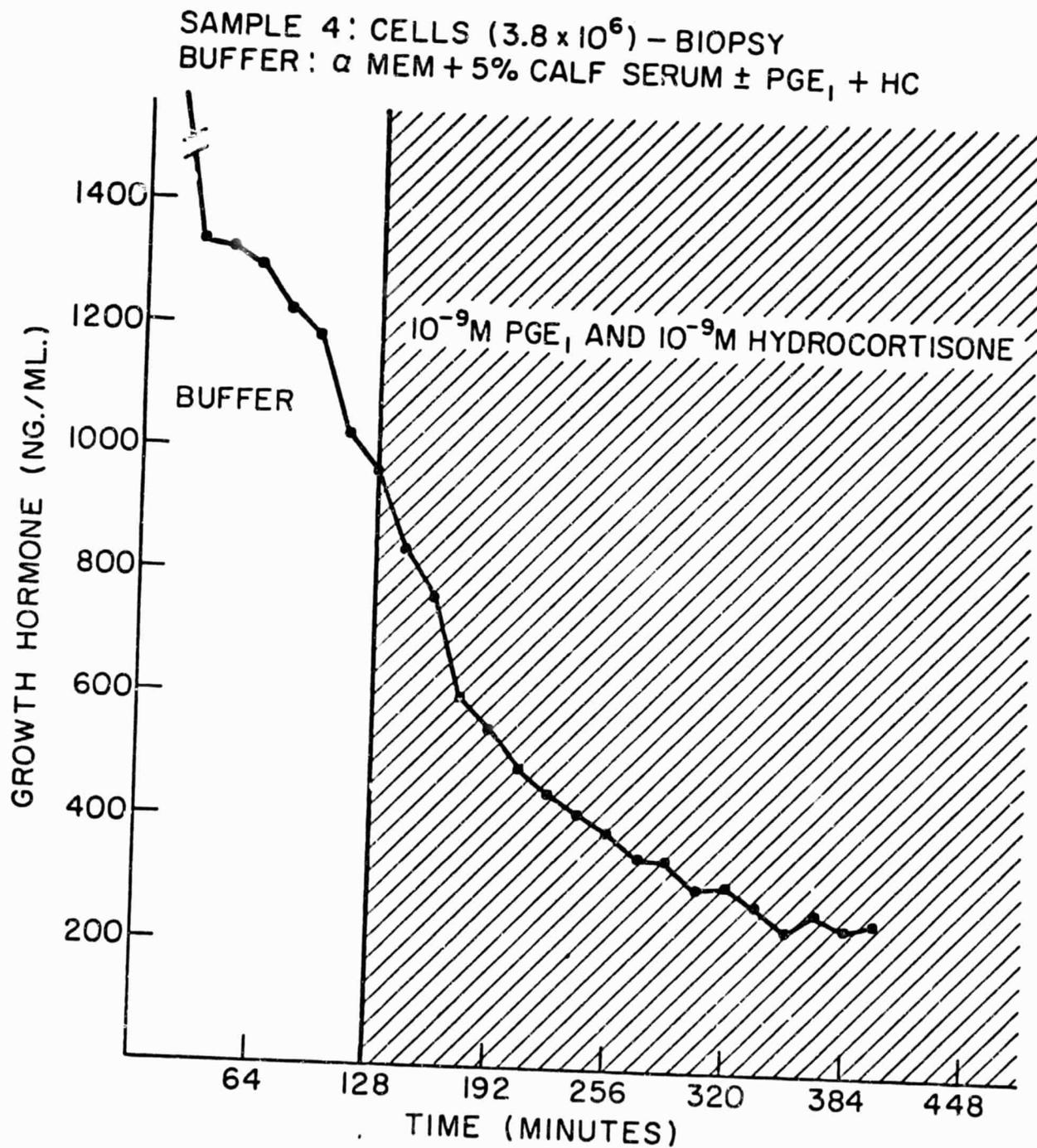
Fig. 10

SAMPLE 5 : CELLS ( $0.5 \times 10^6$ ) - BIOPSY  
BUFFER : 199 + 0.1% BSA  $\pm$  cAMP + PGE<sub>1</sub>



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Fig. 11



result which probably reflects their high secretion rates in vivo. The data generated in Fig. 11 are derived from an aliquot of the same cell sample depicted in Fig. 1d.

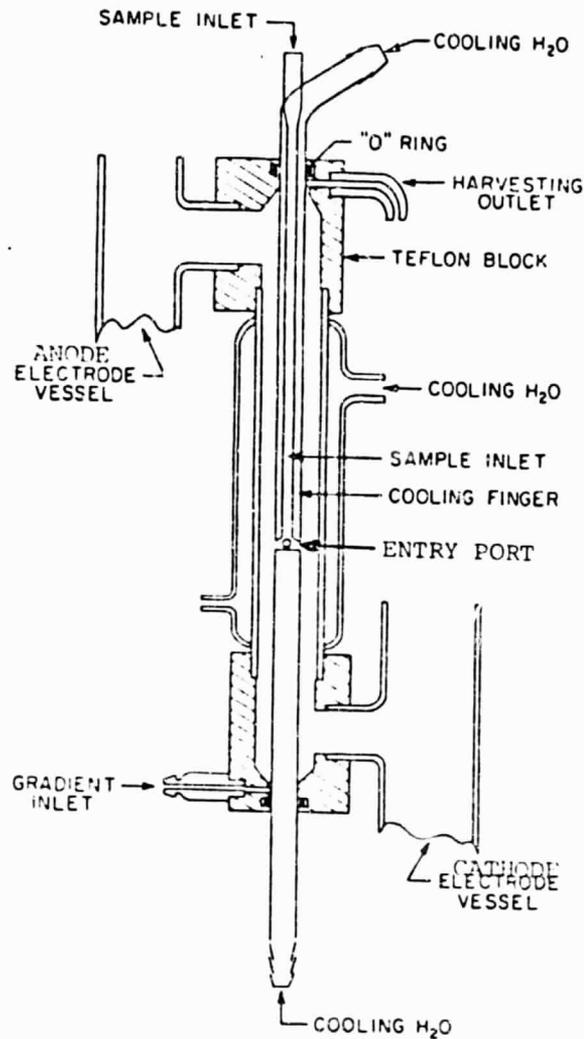
As with the autopsy-cell data (section III-3, above), it seems clear that neither cAMP, HC, PGE<sub>1</sub> nor serum have appreciable effects on the quantity of hGH released from cells prepared from biopsy tissue.

#### IV. Isoelectric Focusing of Rat Pituitary Cells

One of the goals of our contract is the purification of living human somatotrophs utilizing several techniques, including density gradient isoelectric focusing. As a start on this subproject, we have carried out several experiments utilizing rat pituitary cells as the model system.

A modified version of the isoelectric focusing technique described by Boltz et al. (1,2) has been used in attempts to separate rat anterior pituitary cells. The apparatus used in these experiments is shown in Figure 12 (1). The electrode vessels were filled with an anode solution of 1 M H<sub>3</sub>PO<sub>4</sub> and a cathode solution of 1 M NaOH. A "ceiling" solution of 35 ml of light density solution was injected into the column through the gradient inlet. This was followed by a density gradient formed using a gradient-maker to mix a light (8.7% sucrose, 1.0% glucose) and a heavy (10% ficoll, 8.0% sucrose, 1.0% glucose) density solution. Each of these gradient solutions contained 1.0% (w/v) ampholines obtained from LKB and 0.001 mgm/ml Fast Green FCF dye used as a pH marker (3). The density gradient was followed by a "floor" solution of 30% sucrose to finish filling the column. Water was continuously circulated around the column to control column temperature. The electrodes within the electrode vessels were connected to a LKB (2103) power supply. The pH gradient was formed by passing a maximum current of 2 mAmps through the column, voltage varied from 400 volts to 1000 volts at completion. After exposure to this current for 12-16 hours, the dye had focused into a series of narrow bands in the acrylamide plug of the anode vessel and in the

Fig. 12



Glassware for density gradient isoelectric focusing of cells. Shaded blocks are fittings made of machined teflon. The height of the cooling finger is adjustable while the column is filled, so sample can be admitted at any desired pH after the pH gradient is formed. The sidearms of the electrode vessels are filled with 15% polyacrylamide gel plugs molded in position. The ammonium persulfate was ionophoresed from these plugs prior to gradient formation.

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ceiling solution where the pH was  $< 3.0$ . This indicated that the ampholines had completed formation of the pH gradient, and that the column was ready for insertion of the cells for isoelectric focusing.

A 1.2-ml sample of the pH-density gradient was removed from the column with a tuberculin syringe attached to the sample inlet. The pH of that portion of the gradient at the entry port was determined from this sample. If another pH was desired for cell loading, the gradient sample was reinserted, the entry port moved to another position within the column, and the procedure repeated.

Anterior pituitaries were removed from 5-10 Fisher 344 female rats (105-108 days old). They were dissociated for 2 hours with trypsin (1:250, Difco) suspended in Eagles minimum essential medium containing 0.1% BSA (4). The cells were centrifuged, washed once in 5-10 ml of Tissue Culture Medium 199 containing 0.1% BSA, and counted in a Hemacytometer. An aliquot containing  $5-10 \times 10^6$  cells was removed from the cellular preparation and washed a second time. This twice-washed pellet of cells was then suspended in the gradient sample obtained from the column. A total of 0.8 ml of this cellular suspension was returned to the column. The remaining 0.4 ml was used as a control fraction; that is, it was held in a tuberculin syringe at the same temperature as the column cooling jacket. A current of 1000 volts and  $\approx 2$  mAmps was then passed through the column. Every 10 minutes the verticle movement of the cells within the column relative to the entry port was recorded. At the end of the isoelectric focusing (60 - 180 minutes), a solution of 30% sucrose entered the gradient inlet under gravity flow, forcing the column contents up and out through the harvesting outlet. A portion (25-30 ml) of the ceiling solution was removed prior to the collection of a total of 40 2-ml fractions. Each of these fractions corresponded to a verticle distance of 0.5 cm within the column. The pH was determined for each fraction. The control and column fractions were washed twice in phosphate-buffered saline containing 0.1% BSA, and Hemacytometer

cell counts were made for every other fraction. Cellular peaks were then identified, and all remaining fractions within these peaks were counted. Cytospin slides were then made of the control fraction and the pooled cellular peak fractions. These were stained with Herlant's tetrachrome stain for identification of cell types.

Quantitative data relating to cell recovery in four isoelectric focusing experiments are given in Table III. Recovery of cells from the electrophoresis column was  $\sim 50\%$ , regardless of the temperature, time, or pH range of the gradient employed in the experiment. Especially noteworthy is the  $\%$  recovery of cells after sitting in the insertion buffer at the same temperature for the same time as used in the electrophoresis run. It is clear that at insertion pH's which are acid (5.2-5.8), only 50% of the cells are recovered. However, at a higher insertion pH (7.2), cell recovery was considerably enhanced in the control cells (82%), even though recovery from the electrophoresis column run in this same experiment was only 42%. Taken together, these results clearly show that some pituitary cells cannot withstand acidic pH's. Approximately 50% of those that were inserted at pH 7.2 in the electrophoresis column did not survive, presumably because they focused at the acidic pH. We are currently evaluating stained samples to see if the cell loss at acid pH is preferential to 1 (or more) cell types.

Distribution profiles of the cells within the pH gradients after isoelectric focusing are shown in Fig. 13. These profiles show one population of cells consistently focusing at pH  $\sim 5.25$ . Others are usually recovered at pH  $\sim 5.6$ , and some are often found at pH 4.9. These patterns seem to be reasonably independent of the experimental conditions of temperature ( $4^\circ$  vs  $15^\circ$ ) and time (60' vs 180').

A detailed histological evaluation of the cells in the different gradient fractions is being done at the time of writing this report.

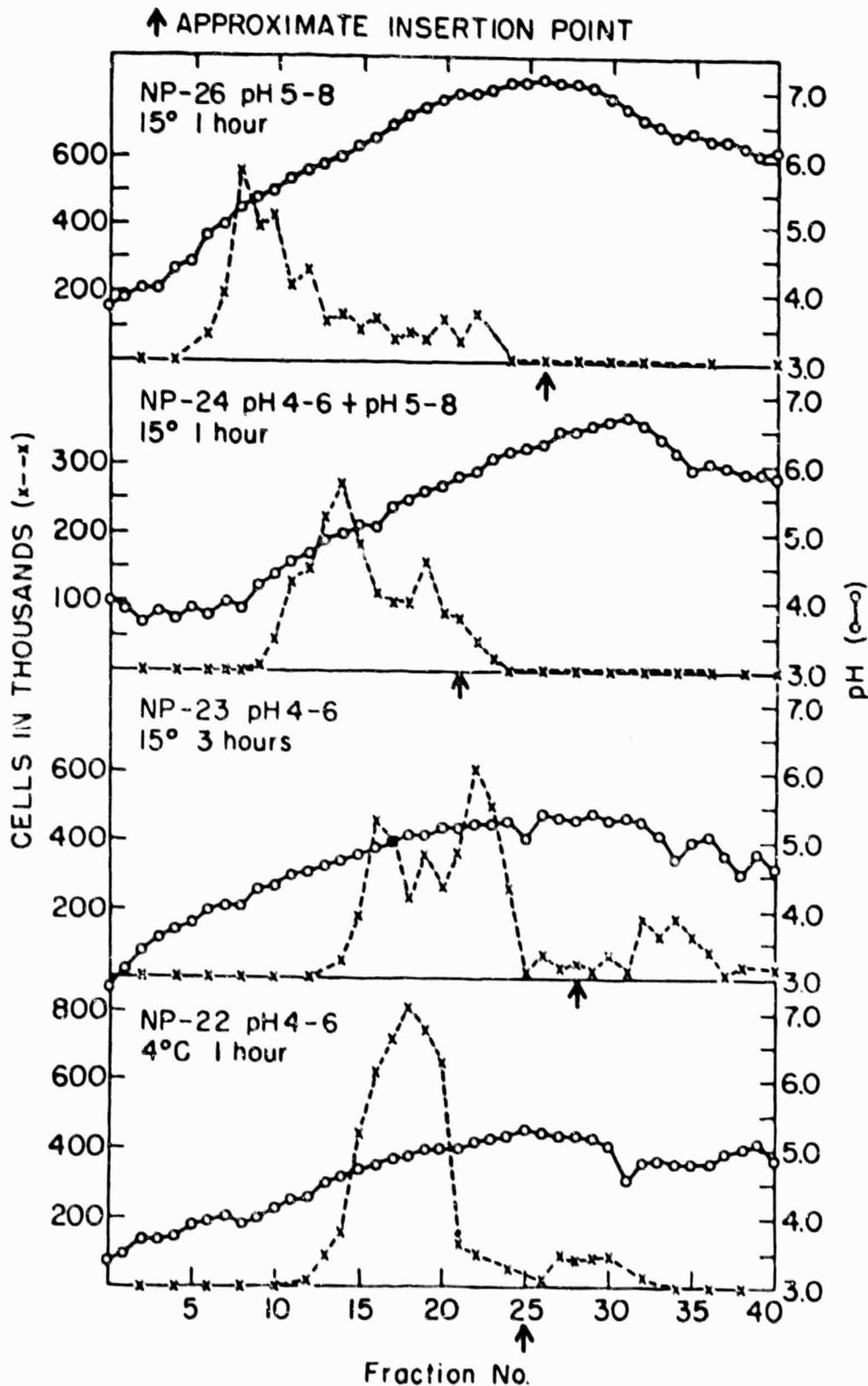
Table III

| Exp. # | Temp.<br>(°C) | Time<br>(min) | Ampholine |     | # Cells<br>Loaded<br>(x 10 <sup>6</sup> ) | % Cells<br>Recovered<br>+ | % Cells<br>Recovered<br>from ++<br>Buffer | Insertion<br>pH |
|--------|---------------|---------------|-----------|-----|---|---------------------------|---|-----------------|
|        |               |               | pH range  | %   |   |                           |   |                 |
| NP-22  | 4             | 60            | 4-6       | 1   | 10.7                                      | 51                        | 41  | 5.2             |
| NP-23  | 15            | 180           | 4-6       | 1   | 10.1                                      | 52                        | 50  | 5.5             |
| NP-24  | 15            | 60            | 4-6       | 0.5 | 4.4                                       | 43                        | 48  | 5.85            |
|        |               |               | 5-8       | 0.5 |   |                           |   |                 |
| NP-26  | 15            | 70            | 5-8       | 1   | 8.3                                       | 42                        | 82  | 7.2             |

<sup>+</sup>Percentage of cells recovered from electrophoresis column.

<sup>++</sup>Percentage of cells recovered from electrophoresis column buffer. These cells were not electrophoresed, but were maintained in the electrophoresis buffer at the insertion pH under conditions identical (i.e., in terms of temperature and time) to those being electrophoresed.

Fig. 13

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#### V. a. Summary

The following statements and conclusions summarize our progress over the period 5/1/78 - 11/1/78.

1. We have set up successful tissue procurement procedures with five collaborating neurosurgeons/pathologists at University hospitals across the country.
2. We have developed methodologies to dissociate human pituitary tissue (obtained either at autopsy or surgery) into single cell suspensions. Using Medium 199 containing 0.5% BSA and 0.3% collagenase I, we can obtain  $\sim 2 \times 10^6$  single cells which can be differentiated at the light microscopic level into various pituitary cell types. No differences in the morphological quality of cells between autopsy and biopsy specimens have been seen thus far.
3. The validity of the Biogel cell column perfusion system for studying the dynamics of GH release has been developed and documented using a rat pituitary cell system. The secretagogues cAMP and  $PGE_1$  are both effective in augmenting GH release (Figs. 2-4).
4. A highly sensitive radioimmunoassay for hGH has been established in our laboratory.
5. On the basis of data presented in Fig. 6, it appears that we can successfully use  $1 \text{ mm}^3$  pieces of human pituitary tissue to monitor release of hGH. Appreciable quantities (300-800 ng/ml) of hGH are released under these circumstances.
6. cAMP does not stimulate hGH release (in fact it may actually suppress release). Prostaglandins and hydrocortisone have no effect on hGH release under the conditions of our experiments.
7. Results similar to those described in #5 and #6 above have been obtained when single cell suspensions; rather than pieces, are used in the Biogel.
8. No functional or morphological differences between cells derived from autopsy vs. biopsy material have been detected thus far.
9. Results from several preliminary experiments dealing with the isoelectric focusing of rat pituitary cells suggest that three cell populations focusing at pH's of 4.9, 5.25 and 5.6 are separable by this technique.

#### V. b. Future Experimental Plans

In the next 6 months of the project we will continue our search for agents which we have reason to believe stand a reasonable chance of augmenting release of hGH from human pituitary tissue in the Biogel system. Many of the experiments will be done with "pieces" and effective stimulatory agents will be subsequently retested on dispersed cells. I feel our tissue procurement program is getting

into "full swing" now, and more samples can be reasonably expected. In this specific regard, I had originally told Drs. Dekker et al., not to provide any material older than 6 hrs. post-mortem. I now feel this requirement was probably too conservative and we have relaxed our requirements to 18 hrs post-mortem. Finally, we recently obtained autoclavable lcc glass microchromatography columns to use for the Biogel experiments. With this new set-up, we should be able to carry out perfusion experiments for a good deal longer, thereby enabling testing for long term effects of the presumed secretagogues.

Among the new agents we propose to test are a) cholera enterotoxin, b) TRH, c) ascites fluid from a rat mammary tumor, b) plasma from acromegalic patients, e) intact rat hypothalami, f) rat hypothalamic extracts and g) purified growth hormone releasing factor (GRF). The use of rat hypothalamic material is suggested since this tissue contains GRF which is not species specific; i.e., it should act on human somatotrophs. In addition, we are negotiating with Drs. Nair and Wilbur of the University of South Carolina Medical School for some of their purified (14 aa) GRF preparation.

With regard to the electrophoresis approach to pituitary cell separation, we propose to evaluate the following modifications: a) column composition (i.e., ampholine concentration, pH range, ficoll concentration, buffer systems), b) insertion pH's (to examine the possibility that proteins are being stripped sequentially from the membrane surface during electrophoresis), and c) the use of density gradient electrophoresis for pituitary cell separation.

1. R. C. Boltz, Jr., P. Todd, R. H. Hammerstedt, W. C. Hymer, C. J. Thompson, and J. Docherty. Cell Separation Methods (1977) 143.
2. R. C. Boltz, Jr., P. Todd, R. A. Gaines, R. P. Milito, J. J. Docherty, C. J. Thompson, M. F. D. Notter, L. S. Richardson, and R. Mortell. J. Histochem. Cytochem. 24:16 (1976).
3. A. Conway-Jacobs and L. M. Lewin. Anal. Biochem. 43:394 (1971).
4. W. C. Hymer, W. H. Evans, J. Kraicer, A. Mastro, J. Davis and E. Griswold. Endocrinology 92:275 (1973).

Addendum to

Mid-Term Report

for

NASA Contract NAS 9-15566

Purification and Cultivation of Human  
Pituitary Growth Hormone Secreting Cells

by

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University Park, PA 16802

November 1, 1978

## I. General Introduction

The need for an adequate supply of human pituitary growth hormone (hGH) for the treatment of children with growth hormone deficiency (GHD) is well recognized. Ms. Alice Maurer, a NASA employee, has recently written an informative and interesting report relating to GHD. For the reader's information, and for purposes of this Addendum, the following excerpts are directly quoted from her report (references omitted)...

The cardinal manifestation of GH deficiency (GHD) in childhood is growth failure. When GHD is congenital, birth weight and length are usually normal but growth retardation becomes apparent within the first or second year of life, the height of these children almost invariably being three standard deviations below the mean for their age. If untreated, severe growth hormone deficiency leads to true pituitary dwarfism, with the individual retaining the stature of a tiny child even in adulthood. Even the less extreme forms of GHD frequently result in some degree of psychological damage to the child who is consistently mistaken for one several years younger than his peers.

Estimates of the prevalence of GHD in children vary widely, from a high of 1 in 60 to 1 in 30,000. Recently, the Scottish Survey of Short Stature reported an incidence of 1 in 4018 for severe growth hormone deficiency in children in three Scottish cities. Whatever the actual prevalence of this condition, most workers agree that hundreds of thousands of children exist who could benefit from GH therapy, and that growth hormone deficiency is probably a severely underdiagnosed affliction.

Ideally, GH replacement therapy should be instituted in infancy, as response then is far better than when treatment is delayed until dwarfism is well established. At any rate, growth hormone must be given before the time when the epiphyseal plates, those actively proliferating cartilage layers of the long bones, become closed. Closure of the epiphyses, a process associated with impending puberty, usually starts at about age 10 and is completed by approximately 18-21 years in males. Characteristically, the growth of a GHD child on GH therapy manifests a "catch-up" curve, being maximal in the first three months of treatment and gradually falling off after that, although growth gains continue throughout puberty if GH administration is maintained. Some workers suggest that intermittent GH therapy, with several months "off" the hormone being alternated with "on" periods, prevents the progressive decline in growth velocity which characterizes continuous therapy. However, most recent studies in this field indicate that continuous treatment is of greater value in achieving the maximum height gain.

Selecting the proper GH dosage for the treatment of GHD is determined not only by therapeutic considerations, but also by the stringent limitations which the scarcity of this substance imposes. Presently,

GH for therapeutic use is derived solely from human cadaver pituitary glands, 80,000 of which are collected annually by the National Pituitary Agency (NPA), a division of the National Institutes of Health. The NPA coordinates the collection and dispersment of human pituitaries for research, diagnostic standardization, and commercial production in the United States. Cal Biochem. Company, this country's only GH supplier, supplements their pituitary supply with material collected in South America. Cal Biochem. does not sell the product to pharmacies or wholesale distributors, but only to the physician who has submitted a formal request along with clinical and laboratory evidence proving growth hormone deficiency in the prospective patient. In addition, GHD patients who are serving as research subjects in an approved project may obtain the hormone free of charge directly from the NPA.

Following the empirically derived recommendations of Raben and Escamilla, the majority of GHD patients receive GH doses of 2 IU three times a week, or 312 IU per year. Frasier has suggested that a lower dose, although perhaps not therapeutically optimal, would more efficiently utilize the limited GH supplies. Based upon measurements of plasma GH levels, he recommends a tri-weekly dose of 0.06 IU per kilogram of body weight. Mason, however, points out that determinations of plasma GH levels are a less desirable index of GH therapeutics than are the levels of somatomedin, and suggests that the future monitoring of GH therapy will be enhanced by the development of sensitive somatomedin receptor assays. Nevertheless, current therapeutic practice generally requires the administration of 312 IU of GH per year, to be continued until a satisfactory height is achieved or the epiphyses close. At a cost from Cal Biochem. of \$300 for 40 IU, this amounts to an annual expense of some \$2240.

These informative data on GHD clearly establish the need and desirability for alternative methods of therapy. One approach, of course, is via recombinant DNA technology. Several laboratories are attempting to get GH genes, which have been introduced into the E. coli genome, translated. If successful, sufficient quantities of GH would then be available to meet therapeutic demand. A second approach could be the implantation of a living human pituitary cells into the brains of GHD patients. The rationale for this approach will become apparent in the following sections of this addendum.

## II. Growth of Hypophysectomized (HYPOX) Rats Bearing Intraventricular Pituitary Cell Implants

About two years ago we discovered that implantation of acutely dispersed pituitary cells into the lateral brain ventricles of hypox rats resulted in partial restoration of growth for periods of 1-3 months. This observation prompted a study of the effect, the result of which will appear in the December issue of Proc. Soc. Exp. Biol. Med. A preprint is enclosed with this addendum. The following statements summarize this study:

- a. growth (% weight gain) of animals bearing  $1 \times 10^6$  cells was similar, during the first 3 week post-implantation period, to sham-hypophysectomized littermates.
- b. growth was proportional to number of cells implanted.
- c. long bone length was significantly greater in the experimental group.
- d. body composition analysis showed that animals in the experimental group put on protein while those in the control group did not.
- e. younger recipients grew better than older ones; cells from pituitaries of older donors gave better responses than equivalent cell numbers from glands of younger donors.
- f. purified somatotrophs gave a positive response.
- g. castration did not affect the response, a result which suggests that anabolic steroids (testosterone) are probably not involved.
- h. immunologically detectable GH was noted in the brains of recipients 1 month post-implantation. However, GH was not detectable in the peripheral serum of these animals.

In a few pilot experiments we noted that implantation of pituitary cells from 1 strain of rats into hypox donors of a 2nd strain resulted in growth for only a short while. We tentatively interpreted this abbreviated response to immune cells in the CSF.

If the pituitary cell implantation approach described in the preprint is to stand any chance of being therapeutically useful for the treatment of GHD patients, the problem of tissue rejection must obviously be addressed. Our approach to this problem is described in the next section.

### III. Growth of Hypophysectomized (HYPOX) Rats Bearing Intracranial Capsules Containing Pituitary Cells

#### A. Diafiber Hollow Fibers: Characteristics

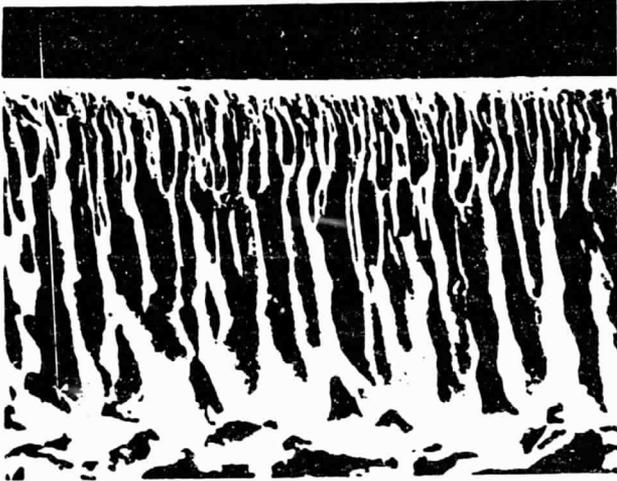
Several years ago Amicon Corporation (Lexington, Mass.) marketed a product which consisted of a very thin anisotropic Diaflo membrane and a thicker spongy layer of the same polymer with increasingly larger openings. These hollow fibers are made of a polyvinyl chloride-acrylic copolymer (XM-50) with an internal diameter of 1000  $\mu\text{m}$  and controlled pore sizes with a nominal molecular weight cut off at 50,000. Examples of their structure are shown in Fig. 1A and B (taken from the Amicon catalogue).

Use of these fibers for the production of artificial pancreas units is becoming more widespread in the research laboratory. Basically, the concept is one of attaching pancreatic islet cells to the surface of an encapsulated fiber which, in turn, is subsequently implanted in a diabetic animal in such a way that blood is flowing through the lumen, of the fiber. Insulin, by virtue of its molecular size, permeates the pore of the hollow fiber and enters the blood stream. However, the animal's antibody producing cells or IgG molecules (150,000 MW) are too large to get at the pancreatic tissue. The usefulness of the idea of an implantable artificial endocrine pancreas unit to restore normoglycemia in diabetic rats has been experimentally verified (e.g., Tze et al., Nature, 264, 466, 1976).

#### B. Capsule Implantation

We reasoned that insertion of pituitary cells into the lumen of the XM-50 hollow fiber, followed by implantation of the fiber into the brain of the

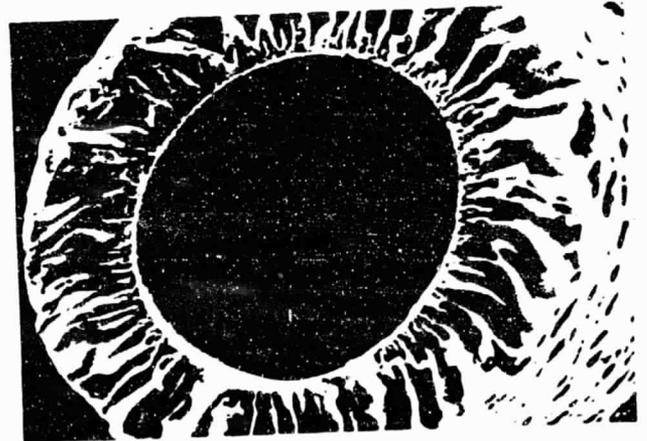
Fig. 1a



DIAFLO MEMBRANE ELECTRON MICROGRAPH (500X). Ultrafiltration "skin" is invisibly thin at the very top. Open-celled substructure is highly permeable to filtrate.

Fig. 1b

DIAFIBER HOLLOW FIBER ELECTRON MICROGRAPH (200X). Ultrafiltration "skin" faces internal channel. Cylindrical substructure opens progressively outward.



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hypox rat, might promote growth in the recipient when pituitary tissue of different species were used (heterografts). Shown in Fig. 2A are fibers implanted into the rat brain coronally (top) or parasagittally (bottom). Even though the capsule takes up a large area of the brain, it has no obvious visible effect on the behavior or physical appearance of the animal. These preparations were done by Dr. R. Page of the Hershey Medical Center. None of the fibers get into the hypothalamus. They often approach a ventricular surface. Shown in Fig. 2B and 2C is the appearance of the capsules removed 1 and 7 days post-implantation, respectively. Note that the pituitary cells are single after 1 day, but after 7 days have aggregated and assume some of the characteristics of an intact pituitary gland. Also note that cells invade the capsule to the point of the limiting membrane. Some have the appearance of polymorphonuclear lymphocytes.

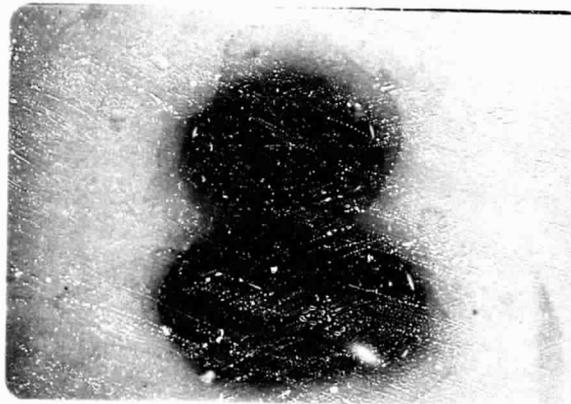
#### C. Growth of Animals Bearing Intracranial Capsules

Shown in Fig. 3 is the growth of hypox ♂ rats over a 55- day period. Some received empty fibers (controls), some received capsules containing  $2.8 \times 10^6$  pituitary cells from the same strain (Sprague-Dawley) and others received capsules containing small pieces of rat pituitary tissue. From previous work (see enclosed MS) we know that the weight gain in controls is attributable to fat. It is clear that pieces/cells of rat pituitary tissue are capable of augmenting animal growth. Presumably rGH (MW 22,000) will pass through the 50,000 MW pores and act (either within the brain itself or in the general circulation) to promote growth via the somatomedin mechanism. Shown in Fig. 4 are the results from a similar experiment in which hypox ♀ rats were used as recipients. The growth promoting effects of the implants are obvious.

The experimental results depicted in Figs. 5 and 6 are interesting in light of the considerations discussed in the beginning of this Addendum. Thus, pituitary cells from a different strain of rats (F-344), sheep, or humans are

Fig. 2

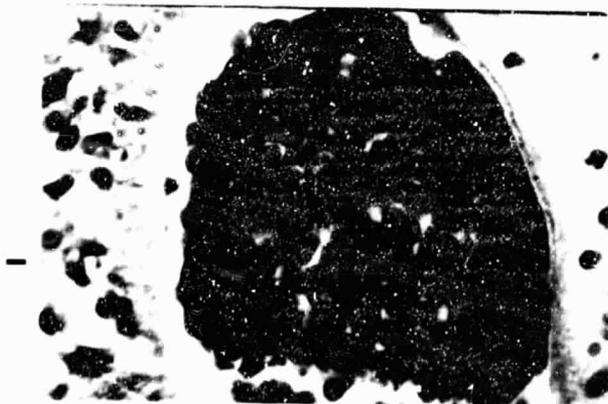
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a

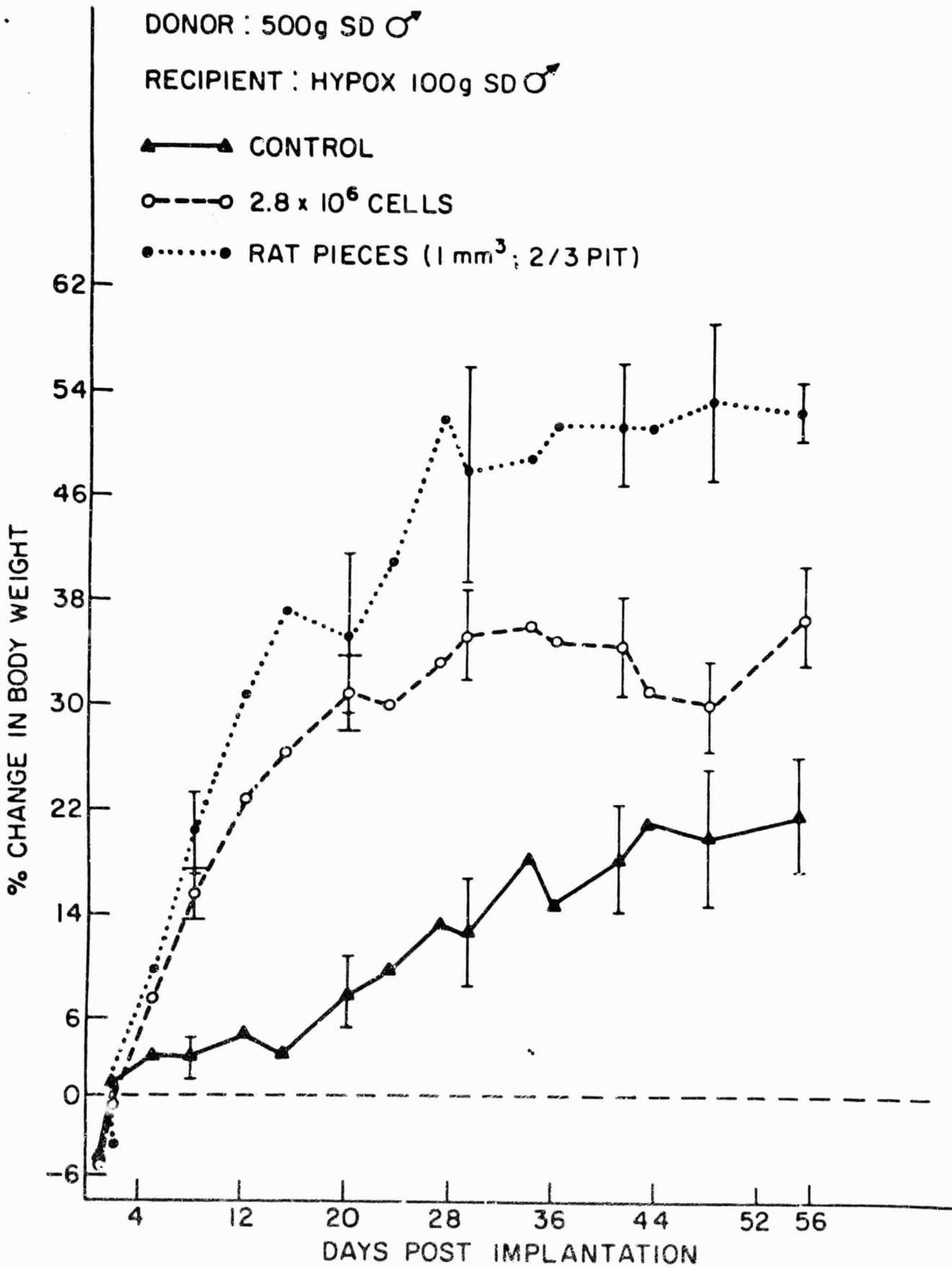


b

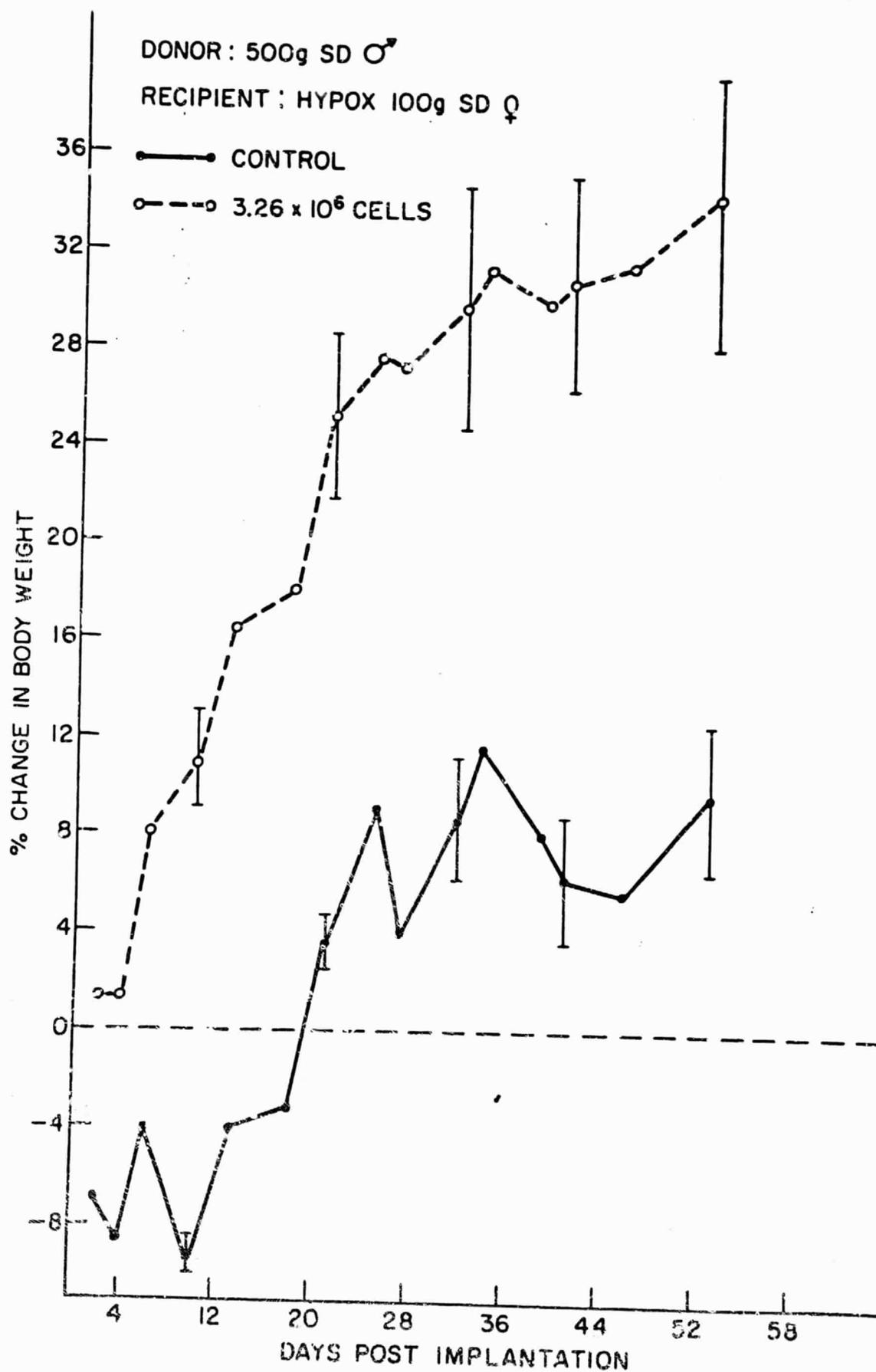


c

A9  
Fig. 3



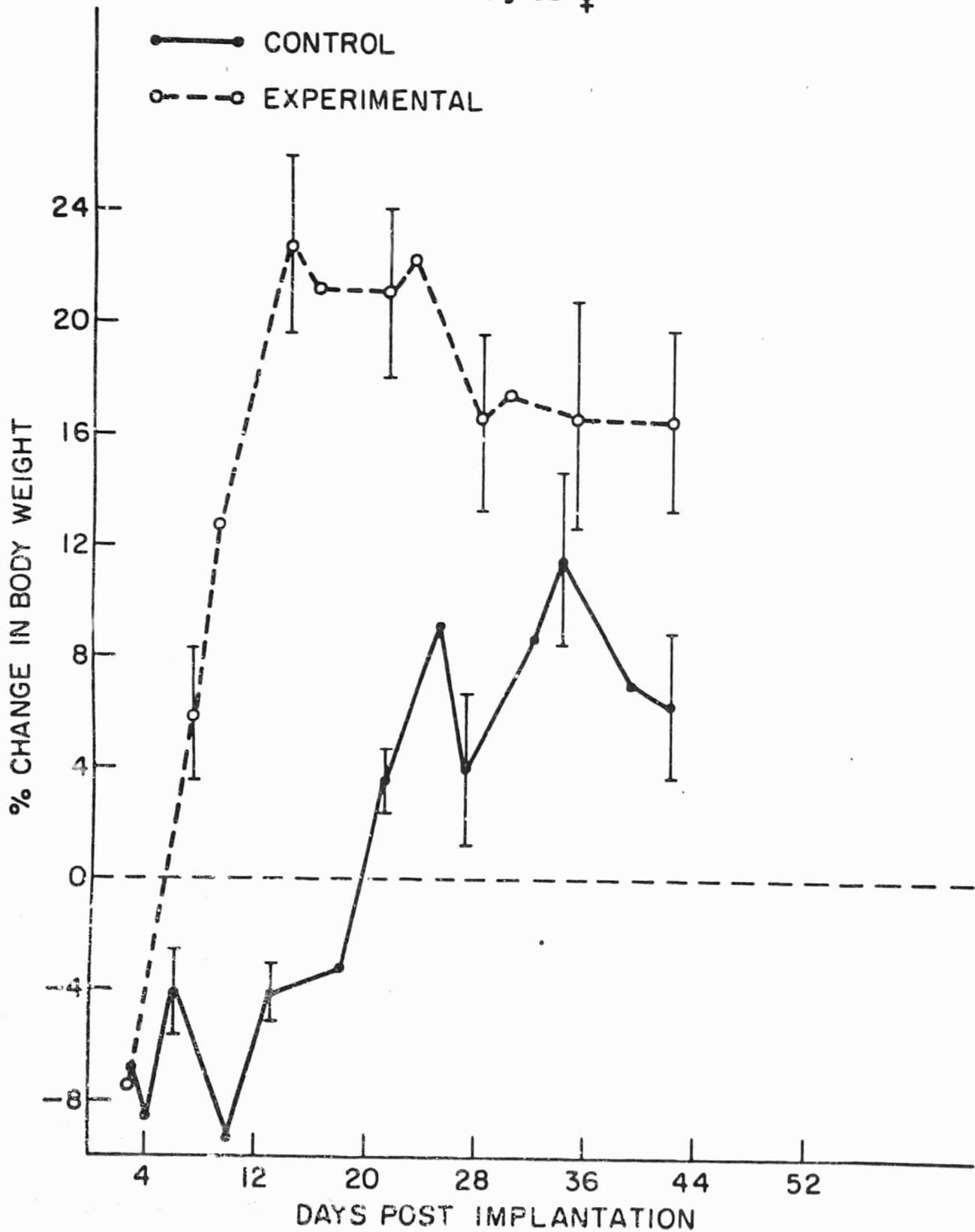
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Fig. 4



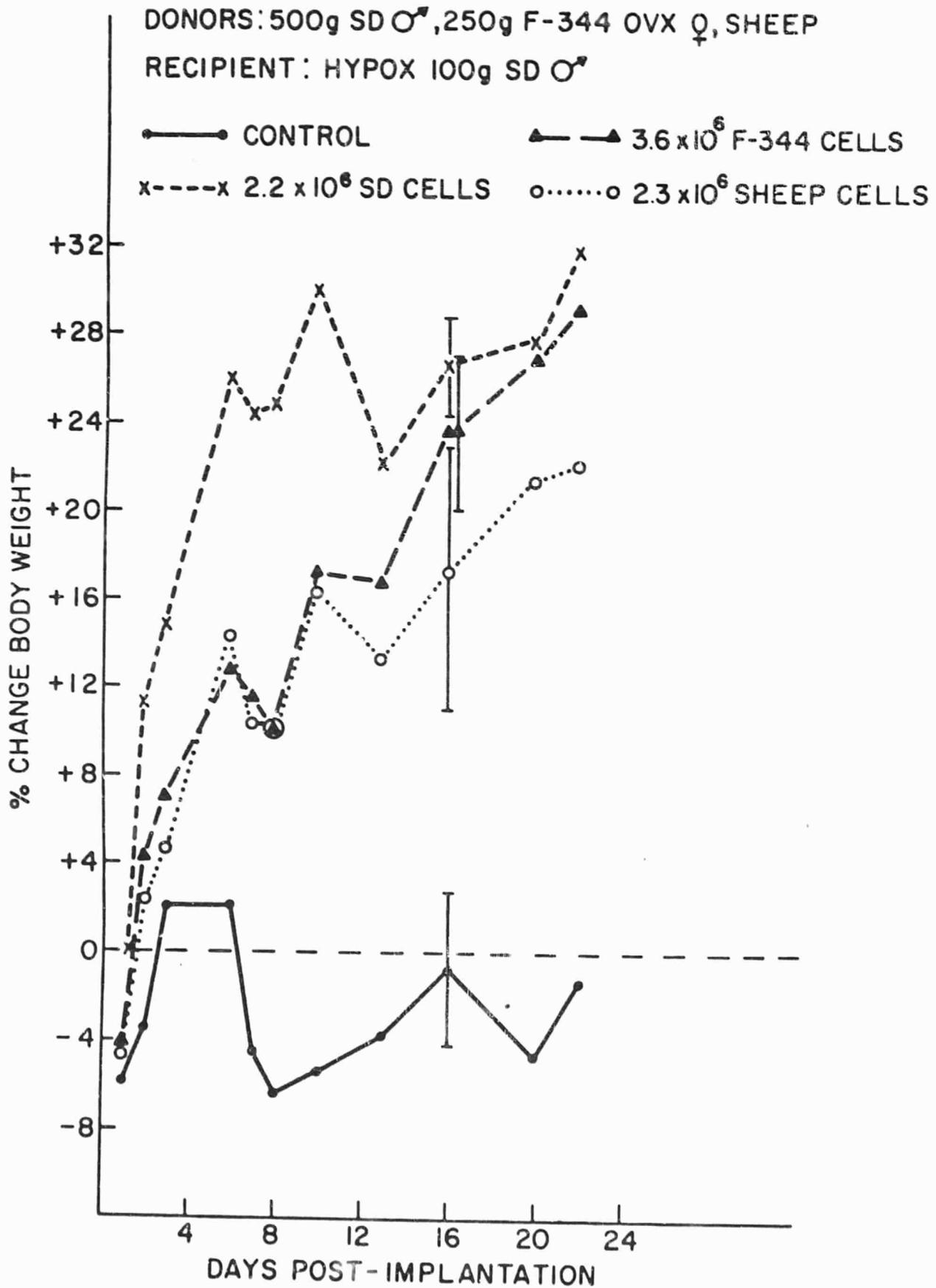
All  
Fig. 5

DONOR : HUMAN PITUITARY (4 HR. POST MORTEM) PIECES

RECIPIENT : HYPOX 100g SD ♀



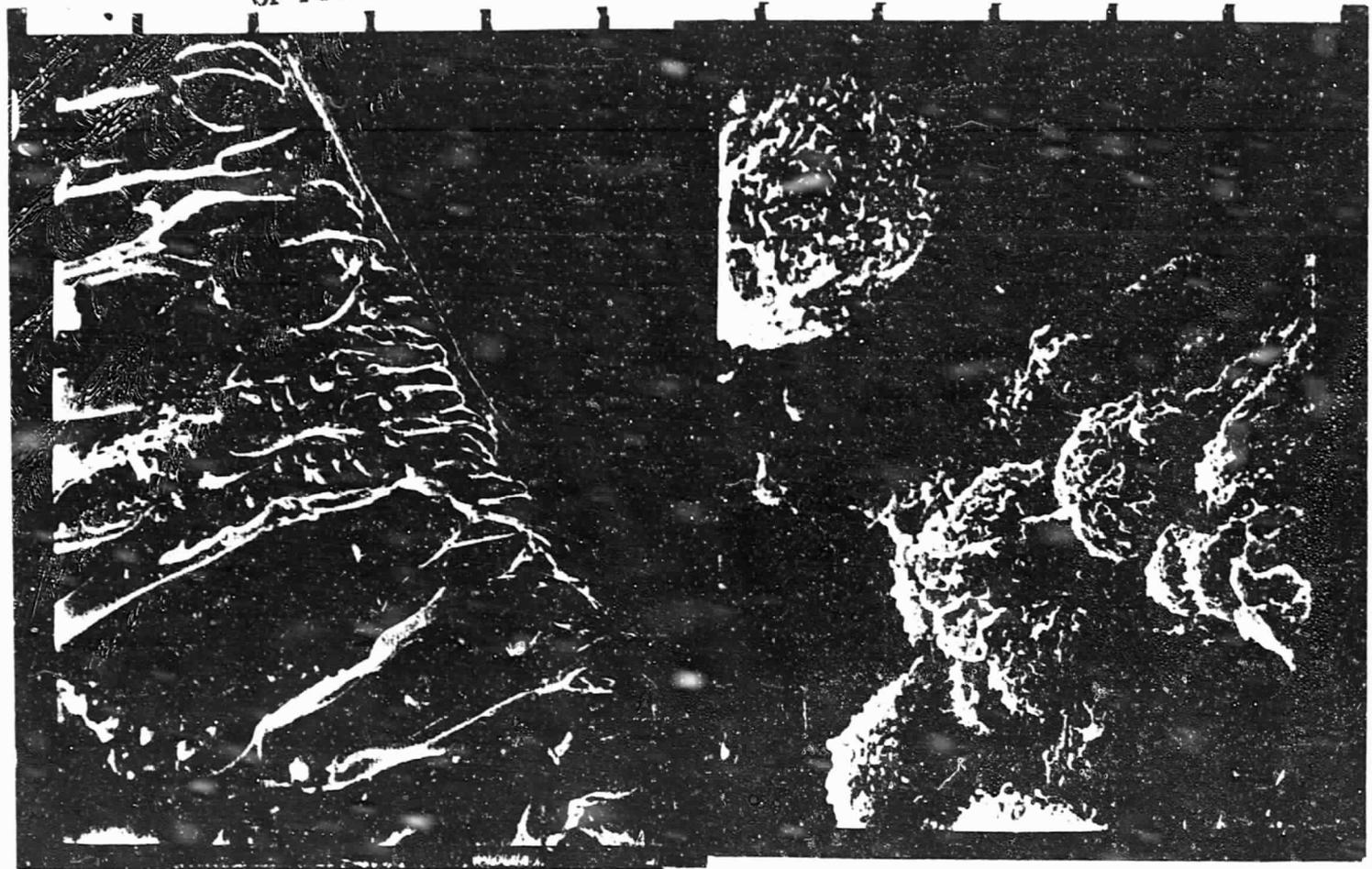
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Fig. 6



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b

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a

c

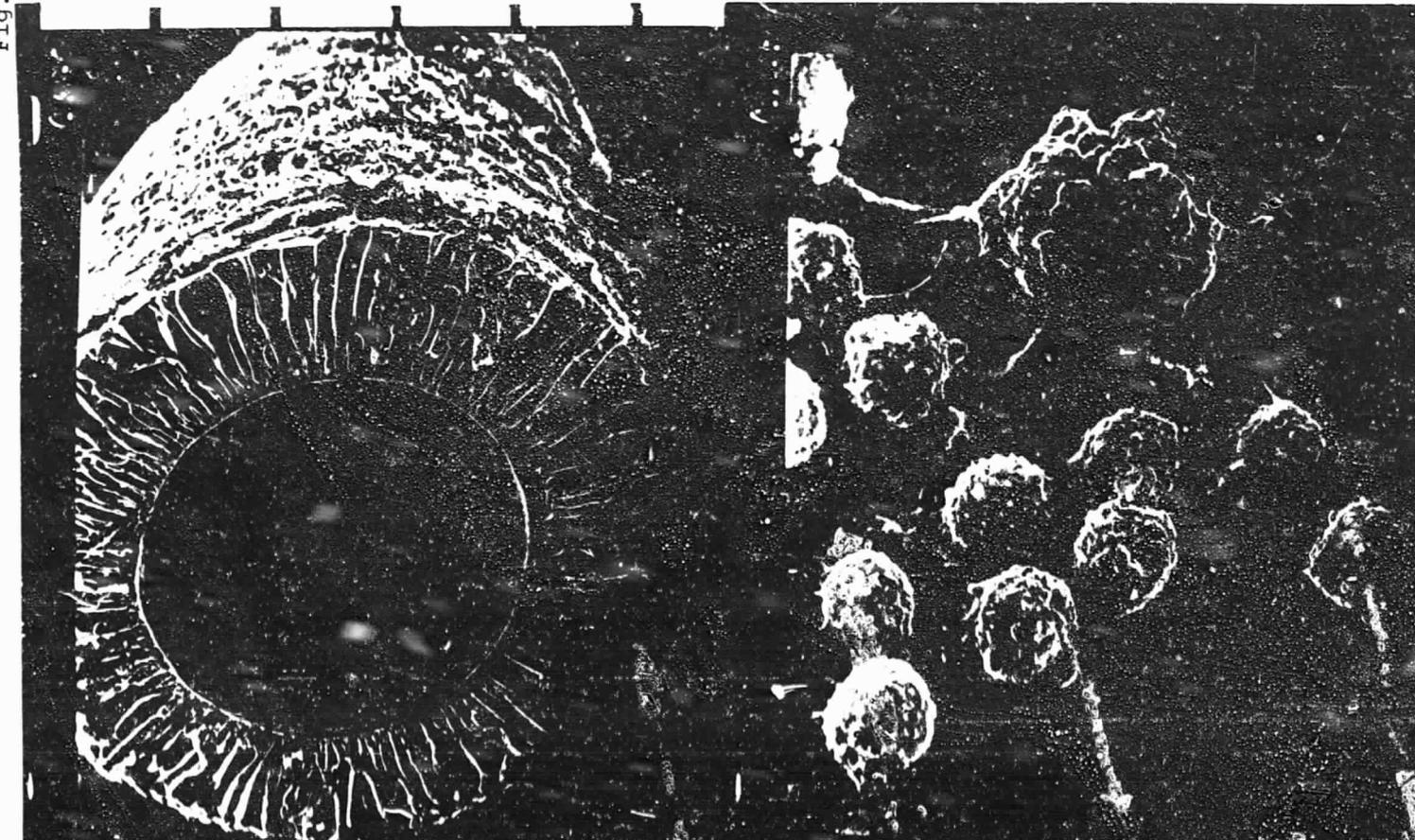


Fig. 7

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all capable of eliciting a positive and significant growth response. Such data argue for the usefulness of this approach in the treatment of GHD patients.

D. Scanning Electron Microscopy of Capsules

Preliminary SEM's of several capsules removed from rats 30-50 days post-implantation are reproduced in Fig. 7A-D. The empty capsule (Fig. 7A) reveals brain tissue on the outer surface, sometimes settling into the crypts of the fiber (Fig. 7B). The nature of these cells is unknown at the present time, but we suspect that they are lymphocytes. The appearance of rat pituitary cells (Fig. 7C) or cow pituitary cells (Fig. 7D) in the lumen reveal a rough surface which is characteristic of functional pituitary cells.

IV. Summary

The need for an adequate supply of hGH and/or new experimental approaches to the therapy of GHD has been described in both the mid-term progress report as well as the Addendum to this report. During the first year of our NASA contract one of our major goals has been, and will continue to be, the search for agents which may be expected to augment release of hGH from the human pituitary somatotroph maintained in vitro. When coupled with our attempts at isolating somatotrophs by electrophoretic techniques, the magnitude of the project is such that a second year of support will certainly be requested. (This was so indicated on pg. 12 of the original proposal). In the original proposal we indicated that part of the effort in the second year would be related to selection of... "histocompatible somatotrophs for potential implantation by using a labeled antibody technique to modify the electrokinetic properties of cells bearing histocompatibility antigens." From the preliminary data presented in Fig. 6 it appears that we may have already discovered a way to overcome the problem of tissue rejection for this particular application (i.e., implantation).

It is important to indicate that the data generated in Figs. 2-7 of the Addendum were done so with no federal support. Money for the purchase of hypox

rats (a substantial figure) was provided to us by Dr. Robert Page, a neurosurgeon at the Hershey Medical Center of The Penn State University. Dr. Page is an expert on mammalian hypothalamo-hypophysial blood vasculature. He has been involved in the experimental design of the implantation studies and is obviously a scientific collaborator on them. Due to circumstances beyond his control, Dr. Page recently indicated to me that he will no longer be able to provide financial support for this project.

This addendum to our mid-term report was included to establish that our in vivo implantation approach is clearly compatible with the in vitro hGH studies currently supported by our NASA contract. Our current thinking is to request, in the renewal application to NASA next May, support for a series of experiments involving:

1. continued in vitro approaches for the cultivation of actively secreting human pituitary somatotrophs (as determined by specific hGH radioimmunoassay);
2. continued electrophoretic approaches to the separation of rat/human somatotrophs; and
3. utilization of the intracranial capsule implantation technique to study the biological activity of human pituitary somatotrophs in vivo.

PITUITARY CELL TRANSPLANTS TO THE CEREBRAL  
VENTRICLES PROMOTE GROWTH OF HYPOPHYSECTOMIZED RATS

CATEGORY FOR "TABLE OF CONTENTS": ENDOCRINOLOGY

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Massachusetts

Sponsor: Harold Schraer

Running Title: Pituitary Cell Transplants

Surgical removal of the adenohypophysis in young animals results in retarded growth as well as decline in peripheral endocrine organ function. Attempts at demonstrating recovery of growth by means of heterotopic hypophysial transplants have met with only limited success (1-5). For example, Halasz and associates reported that transplantation of the whole pituitary gland into the hypophysiotropic area of the brain resulted in partial restoration of growth (3). Growth restoration of a smaller magnitude was observed after transplantation of pituitary glands to such remote sites as the renal capsule (2) or anterior chamber of the eye (1), as well as intramuscular (5) or subcutaneous (4) placements. Gittes and Kastin (5) observed a log dose relationship between growth and number of intramuscular glands and by extrapolation concluded that 750 glands would be required for restoration to normal growth. Meites and Kragt (4), on the other hand, reported partial restoration of growth (46%) in young (37-day-old) hypophysectomized (hypox) ♀ rats bearing a single subcutaneous pituitary for 30 days.

The ease with which pituitary glands can be enzymatically dispersed to yield suspensions of single viable cells is now widely appreciated. In addition to their usefulness for in vitro studies, these single cell suspensions have also been implanted into the kidney capsule or hypophysiotropic area of hypox rats (6,7). On the basis of morphological criteria it was suggested that such transplanted cells retained functionality in vivo.

There is increasing evidence that cerebrospinal fluid (CSF) contains (hypothalamic) neurohormones (8) which may participate in the regulation of pituitary function (9). In the present study, the ventricular system of the brain of hypox rats was therefore chosen as the implantation site for dispersed pituitary cells and the restoration of body growth was used as an index of functionality of the implanted cells.

## MATERIALS AND METHODS

In the usual experiment, hypox Sprague Dawley male rats weighing 80-100 g (~ 30 days old) were purchased from Charles River Breeding Laboratories, Inc., [CD Strain (Outbred Albino), Wilmington, MA] and permitted one week of post-operative recovery. In some cases, sham-hypox littermates were also used. Twenty-gauge hypodermic needles, filed to an unbevelled end 3.25 mm in length and filled with silastic, were stereotaxically implanted into the left ventricle and anchored with acrylic cement according to the procedures of Severs et al. (10). Animals were maintained one additional week prior to cell implantation. During this period, animals showing increases in body weight of > 5% over initial post-operative levels, suggestive of incomplete hypophysectomy, were discarded from the experiment. Anterior pituitaries from donor males of the same strain (CD, 250-400 g, > 70 days) were dispersed in trypsin (11), counted, and resuspended in "mock CSF", consisting of 16 mg dextrose, 176 mg NaHCO<sub>3</sub>, 15 mg KCl, 14.0 mg CaCl<sub>2</sub> (anhydrous), 8.1 mg NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 23.5 mg MgCl<sub>2</sub>·6H<sub>2</sub>O, 13 mg urea, 91 mg NaCl in 100 ml double distilled water. Each animal received a single injection of 10-20  $\mu$ l either "mock CSF" (control) or 1-3x10<sup>6</sup> cells prepared in CSF vehicle (experimental), delivered via the needle of a microliter syringe through the silastic plug of the indwelling cannula. The quantity of cells delivered was equivalent to approximately 1/4-3/4 of a whole pituitary gland. Three to 6 animals were used per group. The animals were maintained with 5% glucose in their drinking water and allowed lab chow ad libitum, under a 12-h light (0600 to 1800) cycle, for periods up to 3 months. They were weighed 3 times per week.

In one experimental series, body composition analysis was done according to the procedure of Hartsook and Hershberger (12). The experimental protocol involved analysis of 12 hypox rats (80-120 g) 2 weeks post-surgery (group A) and 12 hypox littermates which had received either "CSF" or 2x10<sup>6</sup> cells 2 weeks post-surgery followed by a 30-day growth period (group B). Regression analysis

of the body composition data from group A gave the following equations for prediction of initial body compositions of animals in group B: Dry matter =  $0.34 (BW) - 4.77 [r^2 = .95]$ ; Lipid =  $0.1 (BW) + 4.07 [r^2 = .79]$ ; Ash =  $.04 (BW) - .31 [r^2 = .90]$ ; Protein =  $0.2 (BW) - .71 [r^2 = .94]$ . This protocol permitted evaluation of changes in body composition over the growth period.

Growth hormone (GH) was measured with a double antibody radioimmunoassay procedure (sensitivity, 3 ng/ml) with materials provided by the NIAMDD (Rat Pituitary Program). Protein content of brain homogenates was estimated by the Lowry procedure (13).

Growth curves were analyzed by the variance ratio test on double reciprocal plots of log weight gain vs. log time. This transformation yielded linear graphs and randomly scattered residual variance plots. Comparative growth responses at 30 days post implantation, as well as other data (bone lengths, body composition and hormone levels) were analyzed by ANOVA or, when appropriate, Student's t-test.

## RESULTS

Growth response. During the first 3-week post-implantation period, growth of hypox animals bearing  $1 \times 10^6$  cells, expressed as % weight gain, was similar to that of sham-hypophysectomized littermates (Fig. 1). After this time growth tended to plateau (see Fig. 1, Exp. #1 and #2,  $1 \times 10^6$  cells). The growth response was related to the number of cells implanted. At no time did total growth exceed that of the animal with an intact pituitary; however, animals receiving more cells tended to plateau later. A single injection of  $3 \times 10^6$  cells resulted in a doubling of the animals' body weight over a period of 3 months (Fig. 1, insert). Implantation of  $1 \times 10^6$  cells into the ventricles of non-hypophysectomized rats resulted in slightly, but significantly ( $p < .05$ ) depressed growth curves.

There was an increase in tibial and femoral bone lengths measured either radiographically or on bones dissected from the rats at autopsy (see Fig. 2). In both cases bone lengths were significantly ( $p < .05$ ) longer in the experimental group. There was a positive correlation between the two methods of measurement. Actual tibial, femoral, and pelvic lengths were  $31.5 \pm .29$ ,  $26.0 \pm .33$ ,  $29.6 \pm .24$  mm respectively for controls and  $33.8 \pm .88$ ,  $28.9 \pm .24$ ,  $33.3 \pm .44$  mm for experimentals ( $1 \times 10^6$  cells). Correlations with x-rays were  $r = .77$  (tibia),  $r = .84$  (fibula),  $r = .97$  (pelvis).

Body composition. In a separate experiment, 30 day old hypox ♂ rats receiving  $2 \times 10^6$  cells intraventricularly showed weight gains over 30 days of  $22.9 \pm 0.5$  g (SEM) ( $\sim 34\%$  increase in body weight) vs.  $7.5 \pm 1.4$  g ( $\sim 11\%$  increase in body weight) for those receiving "CSF". The increase in the experimental group represented  $14.1 \pm 3.5$  g dry matter of which  $5.0 \pm 1.5$  g were protein,  $8.5 \pm 1.9$  g were lipid, and  $1.1 \pm 0.5$  g were ash. The increase in the control group represented  $2.8 \pm 0.5$  g dry matter of which  $0.1 \pm 0.3$  g were protein,  $2.6 \pm 0.7$  g were lipid and  $0.4 \pm 0.1$  g were ash. These results clearly show that significant ( $p < .05$ ) increases in both protein and fat account for the weight gain in the experimental animals.

Age and sex. Younger recipients showed a better growth response than the older ones (Fig. 3, top). Pituitary cells from older donor animals gave better responses than cells from young animals (Fig. 3, middle). Cells from male donors of different ages gave consistently inferior responses when implanted into young hypox females (Fig. 3, middle vs bottom). This result is consistent with the observation that male rats grow larger than females. Cells from > 70-day-old female donors were as effective as their male counterparts when transplanted into hypox males (data not shown).

Somatotroph implantation. Intraventricular implantation of 630,000 somatotrophs purified to 90% by the method of Snyder *et al.* (14) resulted in a weight gain at 30 days of  $17.4 \pm 3.6\%$  vs  $-1.5 \pm 4.3\%$  for those injected with vehicle ( $p < .05$ ).

Castration. Four groups ( $n=5$ , each group) of hypox recipients, two of which were castrated at the time of pituitary removal, received either  $2 \times 10^6$  pituitary cells or vehicle. Growth (% wt. gain) 30 days post-implantation was as follows: a) castrated animals with cells  $45.8 \pm 10.8\%$ ; b) castrated animals with vehicle  $5.3 \pm 1.5\%$ ; c) non-castrated animals with cells  $69.1 \pm 15.0\%$  and d) non-castrated animals with vehicle  $9.1 \pm 2.7\%$ . Growth of animals at 30 days in both experimental groups was significantly greater than in controls ( $p < .05$ ), but not significantly different between experimental groups.

Brain and blood growth hormone (GH). The levels of GH in homogenates of brains prepared from animals receiving either  $1 \times 10^6$  pituitary cells or vehicle is given in Table I. The data reveal detectable hormone in the brains of the experimental group 30 days post-implantation, but at 1/4 the level detected 12 days post-implantation.

GH levels in the sera of each of the animals in Table I were, in every case, undetectable. Possible reason(s) for this result are currently under study.

Cell placement and viability. In four separate experiments designed to assess requirements of cell placement and viability in relation to the growth

response, the following data were collected (% wt. gain in 30-day-old hypox males one month post-implantation): a)  $1 \times 10^6$  cells - intraperitoneally,  $13 \pm 4\%$ ; b)  $1 \times 10^6$  cells-anterior chamber of the eye,  $7.8 \pm 2.4\%$ ; c) heat-killed ( $56^\circ \text{C}$ , 30 min) cells-intraventricularly,  $4.7\%$ ; and d) a 100,000 x g particle fraction (prepared from  $1 \times 10^6$  cells)  $5.4 \pm 0.3\%$ . None of these responses were significantly different from vehicle-injected controls, but all were significantly lower ( $p < .01$ ) than the response obtained by implanting  $1 \times 10^6$  cells intraventricularly ( $40.6 \pm 4.0\%$ , mean of the four experiments).

Histology. Serial sections of the entire brains of several experimental animals revealed epithelial cells in the 3rd ventricle, lateral ventricles, and subarachnoid space. Since such cells were not found in the sections of the brains of a control animal, it is tentatively concluded that the pituitary cells spread throughout the entire ventricular system.

## DISCUSSION

The key finding in this study is that implantation of pituitary cells into the ventricular system of hypophysectomized rats results in animal growth. This growth is reflected both in increased bone length as well as deposition of total body protein. Our data show that intact cells placed in the ventricles are required to obtain this response since neither cells placed in the anterior chamber of the eye or peritoneal cavity nor heat killed cells or pituitary organelles gave a positive growth response.

The growth response can probably be attributed to somatotrophs in the pituitary cell suspensions for the following reasons: first, implantation of purified somatotrophs gave a positive response; second, the response was obtained in a castrated animal in which influences of anabolic steroids were not present; and third, GH was detected in the brains of rats 30 days post-implantation of cells, but not in vehicle-injected controls (Table I).

The results show that the CSF of the hypox rat provides a suitable functional milieu for maintenance of somatotrophs for at least 3 weeks post-implantation.

## SUMMARY

Implantation of acutely dispersed adenohypophysial cells into the lateral ventricles of hypophysectomized rats resulted in partial growth restoration for periods of one to three months. Weight gain by experimental animals was consistently 20% to 60% greater than among hypophysectomized control rats; the response was related to the number of cells implanted. The weight gain reflected increases of both protein and fat in body composition. A significant increase in long bone lengths was also observed among rats bearing intraventricular cells. Intraventricular implantation of either heat-killed anterior pituitary cells or subcellular organelles, or implantation of pituitary cells in the peritoneal cavity or anterior chamber of the eye, did not promote significant growth in hypophysectomized recipients. The results suggest that transplanted growth hormone-secreting cells are provided with a suitable functional milieu by the cerebrospinal fluid of the hypophysectomized rat.

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Table I. Growth hormone levels (ng GH/mg protein) in brain homogenates prepared from hypox rats previously implanted with  $1 \times 10^6$  pituitary cells (experimentals) or CSF vehicle (controls).

| Treatment                  | Days Post-Implantation      |                |                |
|----------------------------|-----------------------------|----------------|----------------|
|                            | 12                          | 20             | 30             |
| Experimentals <sup>a</sup> | 53.4 $\pm$ 9.0 <sup>b</sup> | 16.1 $\pm$ 4.1 | 13.6 $\pm$ 2.3 |
| Controls                   | 3.2 $\pm$ 3.2               | 0 <sup>c</sup> | 0 $\pm$ 0      |

<sup>a</sup>30-day-old hypox ♂ rats received  $1 \times 10^6$  pituitary cells from 70-day-old ♂ rats.

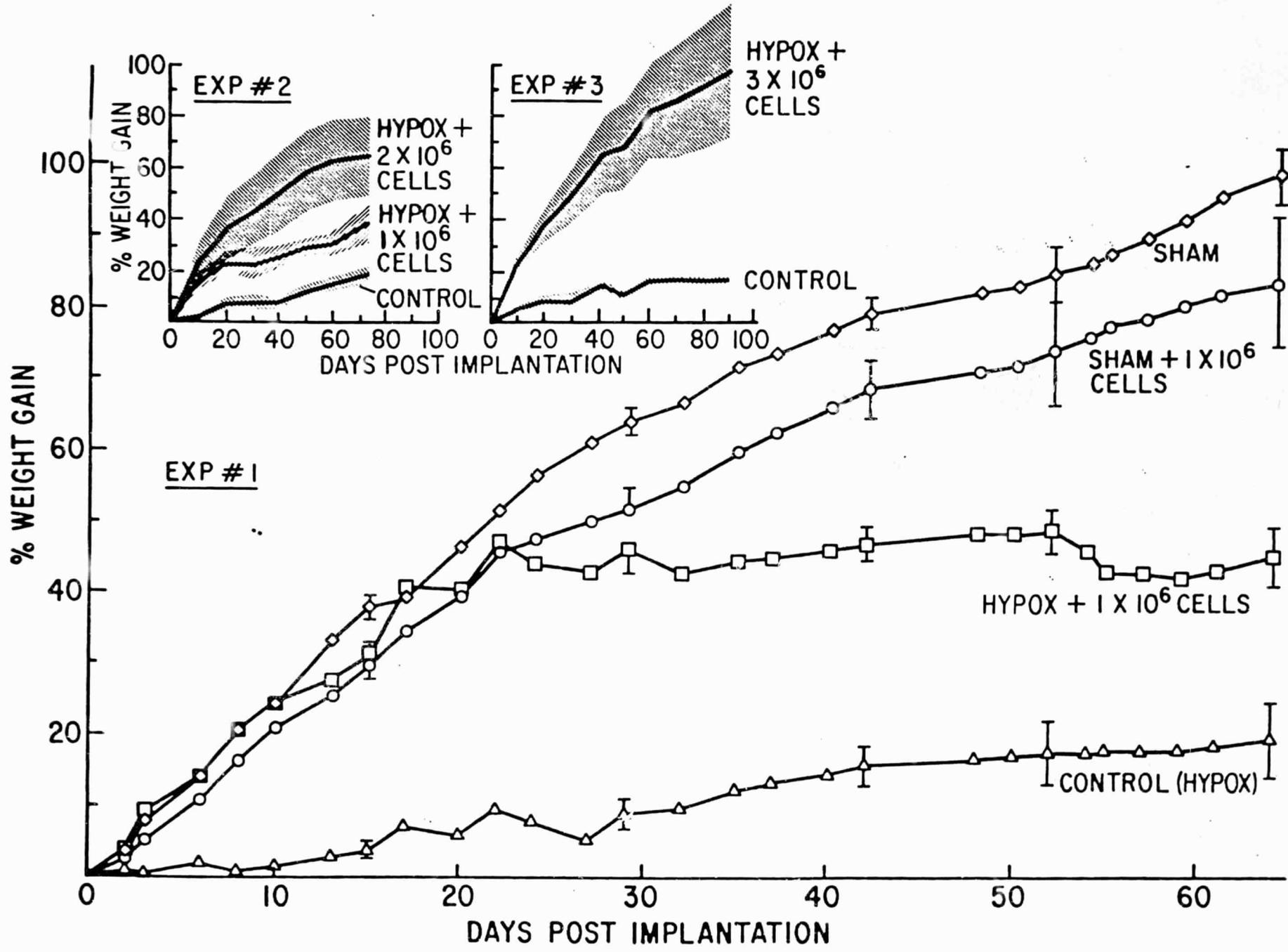
<sup>b</sup>Standard error of mean.

<sup>c</sup>One animal; all other groups had 3-4 animals.

## FIGURE LEGENDS

- Fig. 1. Exp. #1, percent increase in body weight of ~ 30 day male hypox rats receiving a 10  $\mu$ l intraventricular injection of either "mock" cerebral spinal fluid (CSF control) or  $1 \times 10^6$  single pituitary cells from 70-day-old donors (bottom 2 lines) or sham hypophysectomized littermates  $\pm 1 \times 10^6$  pituitary cells (top 2 lines). Each line corresponds to the weight gain of 4 animals; error bars and shading represent  $\pm$  SEM. Effect of implantation of 1x, 2x (Exp. #2) or  $3 \times 10^6$  cells (Exp. #3) on weight gain is shown in the inserts.
- Fig. 2. Radiographs and bones (tibia-lower, femur-upper) from 2 hypox animals 30 days after intraventricular implantation of either  $2 \times 10^6$  cells (left) or "mock" CSF vehicle (right). Scale bar equals 1 cm.
- Fig. 3. Effect of age of recipient at hypophysectomy (top), and age of donor pituitary cells in  $\overset{\uparrow}{\text{O}}$  recipients (middle) or  $\underset{\downarrow}{\text{Q}}$  recipients (bottom) on weight gain. Statistical analysis: top panel: one animal in the 30 day experimental group grew 3x more than the other 3. Analysis of variance (ANOVA) on these data excluding this single animal resulted in significant ( $p < .05$ ) elevations in the experimental groups in all cases. Middle panel: by ANOVA 50 and 70-day-old donor cells caused significant ( $p < .05$ ) growth. Bottom panel: growth, although apparently elevated, was not statistically significant.

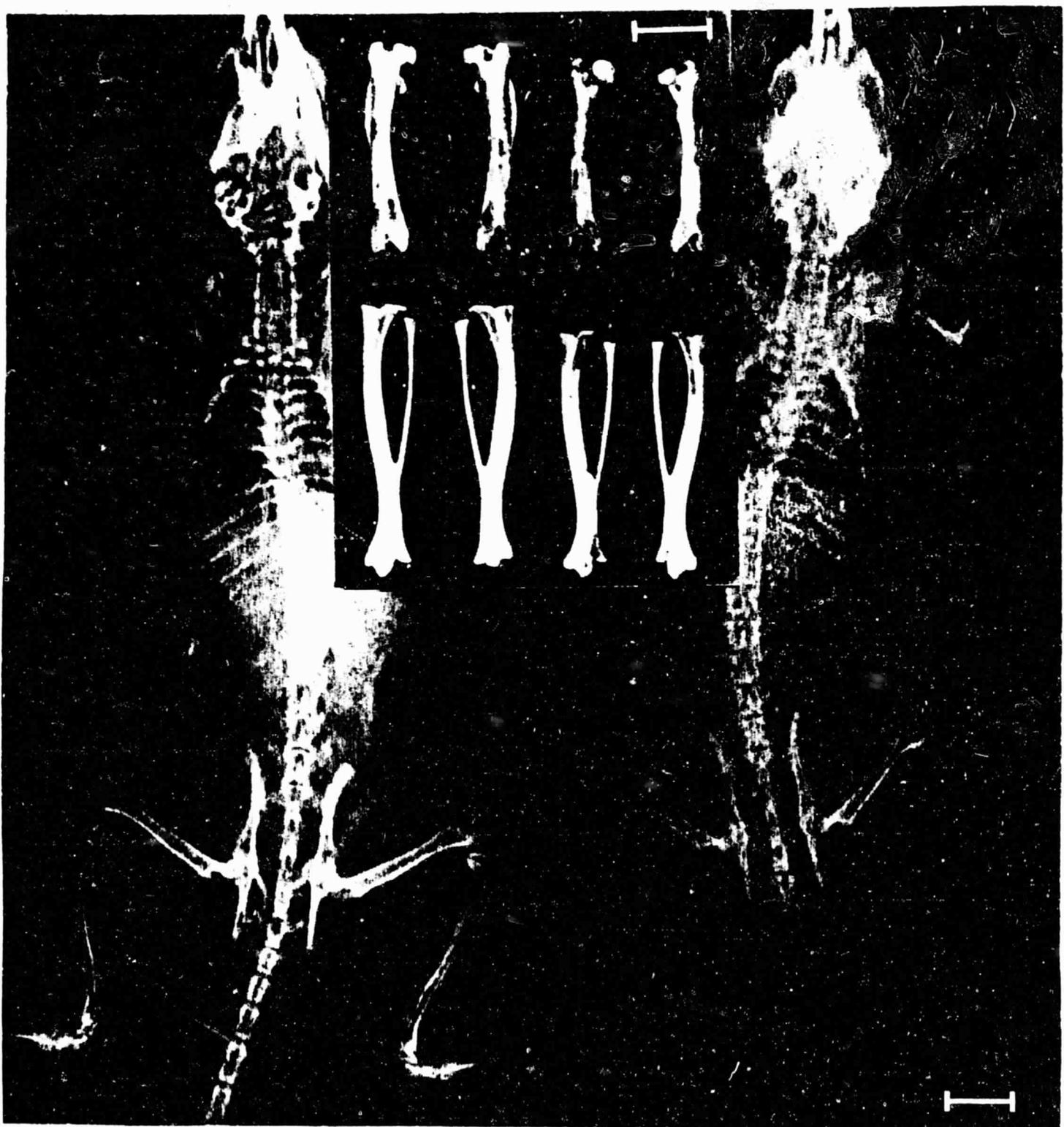
Fig. 1



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