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EXPERIMENT K-6-22

GROWTH HORMONE REGULATION, SYNTHESIS AND SECRETION IN MICROGRAVITY

PART I: SOMATOTROPH PHYSIOLOGY

PART II: IMMUNOHISTOCHEMICAL ANALYSIS OF HYPOTHALAMIC HORMONES

PART III: PLASMA ANALYSIS

Principal Investigator:

R. Grindeland
NASA-Ames Research Center
Moffett Field, California 94035

Co-Investigators:

W. Vale
Salk Institute
LaJolla, California

W. Hymer
Pennsylvania State University
University Park, Pennsylvania

P. Sawchenko
Salk Institute
LaJolla, California

M. Vasques
NASA-Ames Research Center
Moffett Field, California 94035

I. Krasnov
A. Kaplansky
I. Popova
Institute of Biomedical Problems
Moscow, USSR

I. Victorov
Brain Research Institute
Moscow, USSR

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PART I: SOMATOTROPH PHYSIOLOGY

R. Grindeland, W. Hymer, I. Krasnov, I. Victorov, and A. Kaplansky

INTRODUCTION

Muscle atrophy, decreased bone growth and calcium loss occur as a result of long term spaceflight. Since pituitary growth hormone (GH) controls the activity of both muscle and bone, effects of spaceflight on GH cell function have received some attention. For example, in the Space Lab 3 (SL-3) mission of 1985, pituitary glands from rats flown in space for 7 days were enzymatically dissociated into single cell suspensions upon return of the animals to Earth (1). Several structure-function tests were applied to these GH cells to determine if flight had affected their capacity to synthesize and release hormone. Although the percentage of GH cells from the 200 gm rats was not affected, the hormone content per somatotroph was greater in flight cells than in the ground based control cells. In culture, GH cells from the flight animals released about 30% less hormone. Furthermore, when implanted into the cerebral ventricles of hypophysectomized rats, GH cells from the flight group released about 50% less hormone into the recipient host than similarly treated control cells. Taken together, these results suggested that GH cells from the flight animals had experienced a partial shutdown in hormone secretion. Finally, HPLC fractionation of culture media showed that a high molecular weight GH variant, rich in bioactivity, was much less prevalent in the experimental group.

The impact of spaceflight on GH cell function was also addressed in the flight of STS-8 (1983) in which rat pituitary cells were flown in sealed tubes maintained at 37°C in a middeck locker. Upon return to Earth, GH secretion from flight cells was reduced about 20 fold compared to controls. The idea that microgravity specifically and directly affected GH secretion was supported by the finding that prolactin (PRL) release from other cell types in these same suspensions was unaffected by flight (2). Thus results from both space exper- iments offered evidence that GH cell function was attenuated in microgravity.

The Cosmos 1887 mission offered an opportunity to repeat the SL-3 experiment. The design of this new study was dictated by the following considerations: 1) five pituitary glands were available for study, 2) ~2 x 10^6 cells could be prepared from each gland and 3) a number of structure-function tests, each requiring different numbers of cells, were possible. Some of these same considerations arose in the design of the earlier SL-3 experiment. In that study, a decision was made to combine all of the glands from the flight animals prior to tissue dissociation. This strategy was flawed because it precluded statistical comparison of the differences actually found in GH cell function between flight and ground-based controls. Accordingly, a decision was made to modify the experimental design of the 1887 flight so that the GH release from cells of the five individual glands of the flight animals could be compared in a statistically meaningful way with hormone release from cells of the five glands of the ground control animals. Listed in Table 1 are the procedures used to test GH cell structure/ function and the approximate numbers of cells required for each test. In order to accomplish these experimental goals, some cells from each gland were cultured individually while the remaining cells from each gland were then pooled with the others from the same treatment groups for subsequent morphological analyses and transplantation study.

In summary, the overall objective of the 1887 mission was to determine if the results of the SL-3 experiment were repeatable. Additionally, we hoped to be able to extend the earlier findings in

light of the longer duration of flight. Finally, the design of the 1887 experiment was modified to permit statistical analysis of the GH secretion data.

METHOD

1. Tissue Transportation.

Four experiments were done, each involving pituitary glands from 5 animals. These experiments, identified as using glands from flight rats (F-Cos), synchronous controls (S-Cos) or 2 sets of vivarium controls (V-1 Cos and V-2 Cos), were done separately at 2-3 day intervals. Glands from the F-Cos animals were shipped to Moscow from the nominal recovery site in individual teflon vials containing 22 ml of S-MEM + 0.1% BSA + 25mM HEPES (pH 7.4) + 0.2% $NaHCO_3$ + Gentamycin Sulfate (10 µg/ml) + Penicillin (100 U/ml) /Streptomycin (100 µg/ml) (PS) at 37±1°C. The time between tissue removal and arrival in Moscow was 30 hrs. Identical intervals were used for all groups. The cell free transport medium from each vial was frozen and kept at -20C until analysis for GH.

2. Pituitary gland dissociation.

Each gland was minced with a sterile razor blade into n1 mms pieces and dissociated into single cell suspensions in a solution containing 1 ml of S-MEM + 0.3% crude Trypsin (Difco 1:250) + 0.3% BSA + 20 µg DNase (Type I, Sigma) + PS according to the method of Wilfinger, et al. (3). Modifications included hand agitation over 2-3 twenty minute periods with intervening washes. Cells were liberated from the tissue pieces by trituration with a siliconized Pasteur pipette fifty times after each wash step. After each trituration step the remaining tissue pieces were re-exposed to fresh enzyme solution. In all cases complete tissue dissociation occurred after 1 hr. Cells from each gland were numbered consecutively (i.e., rat 1 - 5). There is no relationship between cells from rat #1 in the F-Cos group and rat #1 in the S or V-Cos series. Cell counts and viabilities from each gland were estimated by hemocytometry using phase contrast microscopy (4).

3. Distribution of cells from individual glands

a. Cell blots. Cells (5 x 10^3/blot) from each of the 5 pituitary glands were cultured in humidified dishes on the surface of a 2x2 cm piece of Immobilon membrane in DMEM + 0.025% BSA + 25 mM HEPES for 2 hrs. at 37°C in 95% air:5% CO_2. After this time the media were removed and the paper processed for GH and prolactin (PRL) immunostaining exactly as described previously (5). Specific polyclonal antisera (cross reactivity < 0.3%) to these hormones were used at dilutions of 1:60,000 for GH and 1:80,000 for PRL. Quantitation of secretion from individual cells was accomplished by image analysis using rGH RP-2 or rPRL RP-3 (NIADDK) as reference standards.

b. Intracellular hormone. Cells ($2x10^5$) were incubated overnight at 4°C in 500 ul of 0.01 N $NaHCO_3$ (n=3/gland). After centrifugation (1400 xg, 4°C, 30 min) the supernatant fraction containing extracted hormone was flash frozen and kept at -20°C until analysis. The 1400 xg pellet was sonicated (40% of maximum power for 5 sec.) in 250 ul of $NaHCO_3$ and incubated overnight at 4°C. The amount of GH in the re-extracted pellet accounted for 15% of the total recovered hormone.

c. Cell culture. Cells ($5x10^4$) in 250 ul of either DMEM + 5% calf serum and antibiotics or chemically defined medium [DMEM + 0.2% $NaHCO_3$ + 25 mM HEPES + Insulin 6.25 µg/ml, Transferrin 6.25 µg/ml, Selenium 6.25 ng/ml, BSA 1.25 mg/ml, Linoleic acid 5.4 µg/ml + 0.5 nM $MnCl_2.4H_2O$, 0.5 nM $(NH_4)_6MO_7O_{24}.4H_2O$, 0.25 nM $NiSO_4.6H_2O$, 15.0 nM H_2SeO_3, 250.0 nM $Na_2SIO_3.9H_2O$, 0.25 nM $SnCl_2$, 2.5 nM $Na_3VO_4.4H_2O$, 50.0 nM $CdSO_4$ + Aprotinin 1 TIU/ml + T_3, $1x10^{-7}$ M + Gentamycin sulfate (10 µg/ml) + Penicillin (100 U/ml)/Streptomycin (100 µg/ml)] were cultured in 96 well plates for 3 days in a humidified atmosphere of 5% CO_2:95% air at 37°C.

There were 4 wells/treatment group. After 3 days media were removed from the wells, centrifuged to pellet any non-adherent cells and the supernatant fraction frozen for subsequent hormone analysis. Fresh medium (150 ml) was added to each well. Another aliquot of fresh medium (100 ml) was added to each microfuge tube (no visible cell pellets) and subsequently transferred back to the appropriate well for an additional 3 day culture period. After another 3 days the culture medium harvesting procedure was repeated. GH remaining in the cells after 6 days was measured in $NaHCO_3$ extracts prepared as described previously.

d. Hormone Assays. GH levels in cell extracts and culture media were determined by enzyme immunoassay exactly as described previously (6). In some cases (see text), the biological activity of the GH in the sample was measured using the tibial line assay of Greenspan (7).

e. HPLC. Serum free culture media from 3 and 6 day samples (S-COS, F-CCS) were chromatographed (0.5/ml/min) by gel permeation high performance liquid chromatography (300 SW Protein Pak column; Waters) using 0.1 M phosphate, pH 6.5 in 0.3 M NaCl and 10% 1-propanol buffer system. Fractions were dialyzed against 0.04 M Tris buffer, pH 7.5, lyophilized and reconstituted in PBS prior to immunoassay.

4. Distribution of cells after pooling.

After the cells from each of the 5 animals were used as described in step 3, the remainder were pooled for morphological and implantation studies.

a. Hollow fiber implantation. Cells ($2x10^5$) were loaded into 10 mm long XM-50 Amicon hollow fibers exactly as described previously (8). In the F-Cos experiment, 10 fibers were loaded with medium (1.5 ml S-MEM + 0.1% BSA) and 10 fibers were loaded with cells in the same medium. Each of these fibers was implanted into the lateral cerebral ventricle of a 100 gm male hypophysectomized rat. This experiment was repeated for the S-Cos series. In each case the time between hypophysectomy and hollow fiber implantation was 7 days. Animals were housed in individual filter top cages at 23-24°C. Animals were killed 10 days post implantation and tibia prepared for staining and measurement of the epiphyseal cartilage plate width. Hollow fiber placement was verified by visual inspection of brain slices. Selected organs and muscles of the recipients were weighed at this time.

b. Morphological analyses. Flow cytometric immunofluorescence was done exactly as described previously (9). Basically this procedure involved fixation of cells in suspension ($2x10^5$), permeabilization of cell membranes with Triton X-100, incubation in antiserum to rat GH (1:10,000) overnight, incubation in FITC conjugated second antibody, counterstained with propidium iodide, and analyzed on an Epics V flow cytometer. Immunocytochemistry on cells ($5x10^4$) attached to poly-L-lysine coated coverslips was done using the diaminobenzidene procedure described previously (9).

RESULTS.

Validation of procedures for tissue handling: preflight experiments.

Since the time that elapsed between removal of the pituitary glands at the landing site and arrival in Moscow was expected to be ~24 hrs., preliminary experiments were done at Penn State University to determine the effects of 1) medium composition and 2) temperature on subsequent GH cell function in vitro. In these experiments glands were stored at either 4°C or room temperature in Spinners minimum essential medium (S-MEM) containing 0.1% bovine serum albumin (BSA) or 5% calf serum (CS) buffered with 25 mM HEPES (pH 7.4) and fortified with antibiotics.

In each of two experiments we examined the effects of 26 hr. pituitary gland storage on 1) subsequent cell recovery (after trypsinization); 2) the percentage of GH cells; 3) GH cell size and cytoplasmic granularity; 4) GH release into the storage medium; and 5) GH release from somatotrophs over a 3 day culture period. In each experiment the tests of the GH cells after 26 hrs (experimental) were compared with that of GH cells prepared from glands obtained immediately after sacrifice (control).

The data (Table A1) established that storage of the glands in the simpler BSA medium at room temperature yielded the same number of somatotrophs as the more complex serum-containing medium. They also showed storage at room temperature in BSA gave satisfactory results. The data in Table A2 established that less GH was released from glands stored at room temperature than at 4°C.

The flow cytometer was used to study the effect of storage on GH staining intensity was well as cell size. These parameters were especially important to the 1887 mission since it is possible with this instrument to objectively categorize and study 20,000 cells in a few minutes. The data in Table A3 showed that the level of specific GH staining intensity in somatotrophs from stored glands was not significantly different from cells of non-stored glands. However, storage reduced the forward angle light scatter (FALS) peak. Since this scatter signal is related to cell size, the data established that GH cells of the stored glands were smaller than controls. It is not possible, however, to make definitive statements about the magnitude of the size change-only that it occurred. Finally, the effect of gland storage on GH secretory capacity is offered in Table A4. In both experiments GH release was reduced 31%. Since the absolute levels of released hormone were about the same for all treatment groups, we conclude that secretory rates were not drastically affected by previous storage conditions.

The preflight data in Tables A1-A4 established the feasibility of storing intact pituitary tissue for 26 hrs. prior to enzymatic dissociation to obtain GH cells. On the basis of these preflight data the decision was made to store glands from the 1887 Cosmos mission in S-MEM + 0.1% BSA at room temperature.

The various tests that were applied to pituitary cells from the Cosmos mission (Table 1) covered a time sequence that spanned a few minutes to 7 days. The results of these studies are presented in that same sequence.

RESULTS FROM THE COSMOS 1887 FLIGHT - GROWTH HORMONE

GH released into storage medium.

The concentration and total amount of immunoactive growth hormone (iGH) and bioactive growth hormone (bGH) released into the 30 hr. storage medium (i.e. the time that elapsed from animal kill to pituitary gland dissociation) in both flight (F-Cos) and synchronous control (S-Cos) groups are given in Table 2. While there was a 25% increase in iGH levels released from glands of the F-Cos group relative to the S-Cos group ($p < .05$) there was a 60% decrease in bBH levels released from the flight group ($p < .001$).

GH cell morphology

The yield of cells from individual pituitary glands in the F-Cos group averaged 1.98±0.2/gland. Cell yields from glands in the S-Cos and two vivarium (V-1 Cos and V-2 Cos) were not significantly different. Cell viability, measured by the phase contrast microscope refractility method (4), averaged 93±0.7% in the F-Cos group. Cell viabilities in the other groups were not different.

The percentages of GH cells prepared from glands in the different groups, based on counts of 50,000 cells/treatment group, was not different (Table 3). However, the staining intensity of the GH cells (as measured by the marker index (see legend to Table 3) in the F-Cos group was 2x greater than that of

cells in the S-Cos group. The increased intensity of specific cytoplasmic GH fluorescence is also documented by the morphological appearance of cells shown in Fig.1. These results suggest, but do not prove, that there was more GH/cell in the flight group. GH cells in the F-Cos group tended to be larger, as evidenced by the magnitude of their FALS signal. However the PLS signal, known to reflect cytoplasmic granularity (4), was not different between GH cells in any of the 4 treatment groups.

An example of the appearance of GH cells bound to poly-L-lysine coated slides and stained by the immunoperoxidase method is shown in Fig. 2 (top). These cells have intense dark reaction product (GH) in their cytoplasm. One hundred such cells, selected at random from both the F-Cos and S-Cos groups, were studied by image analysis to determine the area percentage of the cell occupied by specific GH reaction product Fig. 2 (bottom). A majority of the cells in the S-Cos group had 20-40% of their area occupied by GH; this number was 60-80% in the case of F-Cos cells (Fig. 3). Thus specific hormone staining of cells both in suspension (Fig. 1, Table 3) and attached to glass (Fig. 3) showed changes in intracellular patterns of GH in the flight group.

GH cell culture (short term)

The cell blot assay (5) permits quantification of hormone secretion from individual pituitary cells. Shown in Fig. 4 are zones of hormone secretion around GH cells from the F-Cos and S-Cos groups incubated on Immobilon membrane for 2 hrs. at 37°C. These images indicate that considerable variability exists in secretion from individual cells. The average amounts of iGH secreted from 35 single cells selected at random from each of the 5 animals in both treatment groups are shown in Fig. 4. Also shown in this figure are the combined secretion data from all of the cells in the F-Cos and S-Cos groups. The increased iGH release from the F-Cos cells relative to that from the S-Cos cells was statistically significant ($p < 0.05$).

GH cell culture (long term): Immunoactive GH

The issue of in vitro GH secretion from cells of individual flight animals was considered crucial to the overall experimental design and data interpretation. Levels of iGH release from cells of individual animals were surprisingly consistent within any given treatment group (Fig. 5). These data also revealed: 1) that levels of secreted iGH were, in the case of serumless medium, ~70% of those in serum-containing medium; 2) that, relative to the first 3 day culture period, levels of hormone released from cells in the S-Cos group were 2-3x greater during the second 3 day culture period and 3) that flight cells did not show the same corresponding increase in iGH release during the second 3 day culture period. When the data from the cell cultures from individual glands were combined according to treatment group, no significant differences in iGH secretion between flight and synchronous control cells were found in the initial culture period (Fig. 6 Top). However, iGH release from the S-Cos cells was significantly ($p < .05$) greater than that from the flight cells during the second culture period. This difference was found in both serum and serum-free cultures. Shown in Table 4 are results of two independant immuncassays done several months apart. They show that the data obtained in assay #1 was repeatable.

Representative examples of the appearance of cells from both F-Cos and S-Cos groups cultured in serum and serumless media are shown in Fig. 7A and 7B. Cell clumping and fibroblast growth were dominant features of the serum-containing cultures. These features were much less frequent in serumless cultures. There were no obvious differences in either the appearance or growth characteristics between the F-Cos and S-Cos cells in culture.

The results of the 2 vivarium control cell culture experiments, in comparison to the F-Cos and S-Cos cells, are also shown in Fig. 6 (Bottom). In general, iGH release from cells in the 2 vivarium experiments was greater than iGH secretion from either F-Cos or S-Cos cells during the initial culture period. However, this difference was not maintained in the second culture period.

GH cell culture (long term): bioactive GH

The sensitivity of the tibial line bioassay precluded analyses of individual culture wells. Furthermore, since 50-75% of the 250µl in each culture well was used for immunoassay and HPLC analyses we decided that the best way to obtain estimates of secreted bGH was to pool all of the remaining samples within the flight, synchronous, or vivarium groups. That is to say, for example, serum containing and chemically defined media from 3 and 6 day cultures of cells from the F-Cos groups were all combined. This strategy generated four tubes (F-Cos, S-Cos, V-1, V-2), each containing 4.0-4.5 ml. One ml was injected into a hypophysectomized rat (n=4/group) according to the method of Greenspan. The data in Table 5 clearly show that the levels of bGH released from the F-Cos cells in vitro were below the sensitivity of the assay, where as those from the other three groups were sufficient to increase tibial plate width.

Intracellular iGH

The data in Table 6 offer information relative to the intracellular contents of iGH before and after culture. They also show the capacity of the cells to synthesize iGH in culture. In general the results indicate that the flight cells, relative to S-Cos cells, 1) initially contained the same amount of iGH; 2) contained less iGH at the end of culture and 3) synthesized less iGH in culture. Levels of bGH in these samples could not be measured due to insufficient sample volume.

GH release from transplanted cells using hollow fiber methodology (8).

Over a 10 day period, hypophysectomized rats implanted with pituitary cells from the S-Cos group secreted 0.21 µg bGH as estimated by tibial line responses of the host (Table 7). Rats implanted with the same number of cells from the F-Cos group secreted significantly less GH in vivo ($p < 0.05$).

The weights of various organs and muscles of animals receiving cell implants are compared with their corresponding control groups in Fig. 8. Rats implanted with cells from the S-Cos group tended to have greater thymus and testes weights than their controls. Rats implanted with cells from the F-Cos group also showed increases in thymus weight. In addition, livers and muscles of the F-Cos cell receipients tended to be heavier than controls. Except for the livers, these differences were not statistically significant.

HPLC fractionation of serumless culture medium from S-Cos and F-Cos cells.

The percentage distributions of GH in different molecular weight fractions obtained by HPLC size exclusion chromatography are given in Table 8 and a typical protein elution profile shown in Fig. 9. In both the 3 and 6 day culture medium samples, most of the hormone was recovered in a fraction (18-62 kD) that would be expected to contain monomeric GH (22 kD). These distributions were remarkably similar between F-Cos and S-Cos samples. A greater percentage of high molecular weight GH was detected in the 3 day than the 6 day cultures.

RESULTS FROM THE COSMOS 1887 FLIGHT - PROLACTIN

PRL released into storage medium

The concentration of immunoactive prolactin (iPRL) released into the 30 hr. storage medium was not significantly different between F-COS (351±63 ng/ml) and S-COS (307±45 ng/ml) samples.

PRL cell morphology

The percentages of PRL cells prepared from glands in the different groups, each based on counts of 25,000 cells, was ~15% higher in the F-COS group (Table 9). This increased percentage is considered significant. PRL cells in flight group showed slightly less intense PRL immunofluorescence staining than those from S-COS, a result which is probably of marginal significance. Since the FALS channels are on linear scales, the size differences between F-COS and S-COS cells again appears marginal. On the other hand, the increased PLS peak channel in the F-COS group (Table 9) indicates that the cytoplasmic "granularity" of PRL flight cells is greater.

PRL cell culture (short term)

The cell blot assay (5) permits quantification of hormone secretion from individual pituitary cells. Shown in Fig. 10 are zones of hormone secretion around PRL cells incubated on immobilon membrane for 2 hrs. at 37°C. These images are representative of many, and document that considerable variation exists between cells. The average amounts of iPRL secreted from 35 single cells selected at random from each of 5 animals in both treatment groups are shown in Fig. 10. The solid bars in panels E and F depict the magnitude of the average amount of PRL secreted from the 5 rats in F-COS and S-COS groups. On average, the increased release of iPRL from F-COS cells relative to S-COS cells was statistically significant.

PRL cell culture (long term)

Levels of PRL released from pituitary cells of individual rats tended to be surprisingly consistent within any given treatment groups (Fig. 11). These data also showed that about 5x more PRL was released from cells cultured in serum containing vs chemically defined media and that the levels of secreted hormone were always significantly higher in the S-COS groups (Fig. 12).

Intracellular PRL

The levels of intracellular PRL in F-COS, S-COS and V-COS groups before and after culture were not significantly different (Table 10). Furthermore, cells cultured in serum-containing media contained n3 times more hormone at the end of 6 days than counterparts cultured in chemically defined medium.

DISCUSSION

The objectives of the 1887 mission were a) to determine if the results of the SL-3 pituitary gland experiment (1) were repeatable and b) to determine what effect a longer mission would have on the rat pituitary gland GH "system". In the 1887 experiment two issues were considered especially important. First, it was recognized that cells prepared from individual rat pituitary glands should be considered separately so that the data from the 5 glands could be analyzed in a statistically meaningful way. Second, results of the SL-3 flight involving the hollow fiber implant and HPLC GH-variant experiments suggested that the biological activity of the hormone had been negatively affected by flight. The results of the 1887 experiment documented the wisdom of addressing both issues in the protocol. Thus, the reduction in secretory capacity of flight cells during subsequent extended cell culture on Earth was documented statistically (Fig. 6) and thereby established the validity of the SL-3 result. The results of both flight experiments thus support the contention that there is a secretory lesion in pituitary GH cells of flight animals.

The issue of the biological (vs immunological) activity of the GH molecule, as it might be affected by flight, is complicated. Certainly this interesting problem is not well understood on Earth. The large discrepancy between activities of GH measured by bioassay vs immunoassay was first described by Ellis and Grindeland in 1974 (10). Since then progress has been slow and hampered by lack of

availability of sensitive GH bioassays. In this context, it is relevant that the GH cell hollow fiber implant method (8) and 3T3 cell assay (11) are established GH bioassays in the literature. Indeed, both were used in the SL-3 experiment and together indicated that release of a high molecular weight GH variant, rich in 3T3 bioactivity, was compromised as a result of spaceflight (1). It would have been desirable to perform 3T3 assays on 1887 samples; unfortunately this assay lost reliability in the years following the SL-3 flight. Thus we were forced to rely solely on the tibial line bioassay whenever possible in the 1887 tests.

Perhaps the most interesting finding to emerge from all of the 1887 pituitary data was the marked and consistent inhibition in release of bGH from the flight cells. The magnitude of this suppression was always greater than that measured by GH immunoassay. Sometimes the responses were completely non-parallel. In one instance, for example, release of iGH was significantly elevated in F-COS samples while the release of bGH was significantly depressed (Table 2).

The relationship between timing of the post-flight tests (SL-3 and 1887) and the secretory responses is depicted in Fig 13. In sum, these data indicate the importance of measuring bGH since the most dramatic differences between flight and control cells were found using GH bioassay.

What is the relationship between bioactive GH and the pituitary gland GH "system"? Several years ago it was shown that two subpopulations of GH cells in the rat pituitary could be separated on the basis of differences in density (12). Type I cells were lightly granulated and less dense (1.06-1.07 g/cm^3) than type II cells (>1.07 g/cm^3) which were laden with secretion granules. Later we found that the biological/immunological activity ratio of the GH released from type II cells in culture was consistently 4-5x, whereas that from band I cells was <1 (13).

More recently, other experiments support the hypothesis that high molecular weight S-S aggregates of the monomeric GH molecule, released specifically from type II cells, could represent the bioactive form of the GH molecule (14). Given these data, we would speculate that microgravity specifically affects type II somatotrophs in their ability to release bGH. Clearly, however, the suppression is not 100%! The mechanism of suppression is unknown, but both flight experiments offer important clues. First, when GH cells from flight animals are transplanted to a GRF-rich site (i.e. ventricles of the hypophysectomized rat), they do not respond as well to peptide stimulation. This may reflect a post flight receptor defect in a specific GH cell subpopulation that does not readily recover. Second, image analysis of F-COS GH cells showed that the hormone occupied more cytoplasmic area. A "relaxation" of the network supporting the secretory granules might lead to the result obtained in Fig. 3. Whether such data could be interpreted to reflect a microgravity effect on microtubules in somatotrophs is attractive, but of course highly speculative at this stage. Third, increased immunofluorescent staining of GH cells in the flight group (Fig. 1) could reflect a flight induced change in packaging of the GH molecules within the secretory granule. We speculate that looser packing in the crystal structure of the granule might account for a) less S-S bridging between molecules (thereby increasing sites for antibody recognition) and therefore b) less bioactivity of the GH molecules liberated from the secretory granules on secretion from the cell.

Given the obvious dissimilarities in the experimental conditions between the two missions (animal strain, diet, duration of spaceflight, interval between return to Earth and preparation of pituitary cells), it is remarkable that the results between the two experiments are as similar as they are. For convenience, these are summarized and compared in Table 11.

Prior to the 1887 flight, relatively little was known about the effect of microgravity on rat PRL cell function on return to Earth. Although no data were collected concerning in vitro PRL secretion from cells of the SL-3 flight rats, flow cytometric immunofluorescence studies on these cells had

shown that microgravity exposure for 7 days had little, if any, effect on PRL cell number (1). This was clearly not the case in the 1887 experiment since the percentage of PRL cells was significantly increased (Table 9) and PRL secretion in vitro was significantly decreased (Fig. 12). Although these responses in some ways parallel those obtained with GH cells, there are more ways in which they do not. Thus, comparison of the effects of flight on GH vs PRL cells clearly indicate that cell number and secretion responses are very different. Of course the mechanisms underlying such changes are unknown. However, it is tempting to speculate that these may be unique microgravity-induced effect(s) within each pituitary cell class.

It is worth noting that stress is a well-known stimulus for PRL release. Since PRL levels in the 30 hr. storage medium were not different between F-COS and S-COS cells, it is tempting to conclude that the flight animals were not unduly stressed.

One of the more striking differences between pituitary cells of the SL-3 vs the 1887 rats was the marked discrepancy between percentages of GH and PRL cells. Since different rat strains and diets were used in these two experiments, it is probable that disparate GH/PRL cell ratios resulted. Previous studies in our laboratory document the plasticity of PRL cells in terms of cell division. We speculate that the increased percentages in PRL cells in the F-COS pituitaries reflects the cumulative effects of altered environment, diet, and rat strain. It would be interesting to see if cell division rates in PRL cells are indeed different in microgravity.

The primary objective of both missions was a clear definition of the effect of spaceflight on the GH cell system. There can no longer be any reasonable doubt that this system is affected in microgravity. One explanation for the reason(s) underlying the better known effects of spaceflight on organisms, viz. changes in bone, muscle and immune systems may very well rest with such changes in bGH. In spite of the fact that rats in the Cosmos 1887 flight were on Earth for two days after flight, our data show that the GH system had still not recovered from the effects of flight. Many questions remain. One of the more important concerns the GRF responsiveness of somatotrophs after flight. This will be tested in an upcoming experiment.

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REFERENCES

1. Grindeland, R., W. C. Hymer, M. Farrington, T. Fast, C. Hayes, K. Motter, L. Patil and M. Vasques. Changes in pituitary growth hormone cells prepared from rats flown on Spacelab 3. Am. J. Physiol. 252:209-215, 1987.

2. Hymer, W. C., R. Grindeland, C. Hayes, J. W. Lanhan, and D. Morrison. Life Sciences, Biotechnology, and Microgravity. Aerospace Century XXI, Vol. 64, Advances in the Astronautical Sciences, Univelt, Inc. San Diego, CA 1987, pp. 1333-1345.

3. Wilfinger, W. W., W. J. Larson, T. R. Downs, and D. L. Wilbur. An in vitro model for studies of intracellular communication in cultured rat anterior pituitary cells (Abstract) Tissue Cell 16:483, 1984.

4. Hatfield, J. Michael and W. C. Hymer. Flow cytometric analysis and sorting of live male rat anterior pituitary cell types by forward angle and perpendicular light scatter. Endocrinology 119:6, pp 2670-2682, 1986.

5. Kendall, M. E. and W. C. Hymer. Cell Blotting: A new approach to quantify hormone secretion from individual rat pituitary cells. Endocrinology. 121:6, 2260-2262, 1987.

6. Farrington, M. A. and W. C. Hymer. An enzyme immunoassay for rat growth hormone: Applications to the study of growth hormone variants. Life Sciences, Vol. 40:2479-2488, 1987.

7. Greenspan, F. S., C. H. Li, M. E. Simpson, and H. M. Evans. Bioassay of hypophyseal growth hormone: the tibial test. Endocrinology 45:455-463, 1949.

8. Hymer, W. C., D. Wilbur, R. Page, E. Hibbard, R. Kelsey, and J. M. Hatfield. Pituitary hollow fiber units in vivo and in vitro. Neuroendocrinology 32:339-349, 1981.

9. Hatfield, M. and W. C. Hymer. Flow cytometric immunofluorescence of rat anterior pituitary cells. Cytometry 6:137-142, 1985.

10. Ellis, S., and R. E. Grindeland. Dichotomy between bioassayable and immunoassayable growth hormone. In Advance in Human Growth Hormone Research, edited by S. Raiti, Washington, D.C.: USA Govt. Printing Office, 1974, pp. 409-433 [DHEW (NIH) 74-612].

11. Nixon, T. and H. Green. Contribution of growth hormone to the adipogenic activity of serum. Endocrinology 114:527-532, 1984.

12. Snyder, G., W. C. Hymer, and J. Snyder. Functional heterogeneity in somatotrophs isolated from the rat anterior pituitary. Endocrinology 101:3, pp. 788-799, 1977.

13. Grindeland, R.E., W.C. Hymer, P. Lundgren, and C. Edwards. Differential secretion of bioassayable growth hormone by two types of rat somatotrophs. The Physiologist 25:262, 1982.

14. Farrington, M. and W. C. Hymer. Characterization of high molecular weight aggregates of rat GH. Abstract for the 70th annual meeting of the Endocrine society, New Orleans, LA, June 1988.

APPENDIX

TABLE A1

EFFECT OF STORAGE CONDITIONS (26 HRS) ON CELL YIELD AND SOMATOTROPH PERCENTAGE

TREATMENT	CELLS RECOVERED/GLAND ($x10^6$)	% CELL RECOVERY	% SOMATOTROP
EXP # 1			
CONTROL (NO STORAGE)	3.6±0.3++	-	27.6±3.6
EXPERIMENTAL (26 HRS)			
MEM + 0.1% BSA - RT+++	2.1±0.1	58	33.9±2.7
MEM + 0.1% BSA - 4°C	2.5±0.2	70	31.4±1.6
√MEM + 5% CS -RT	1.4±0.2	40	16.1±4.5
√MEM + 5% CS - 4°C	2.5±0.3	71	32.5±3.3
EXP # 2			
CONTROL (NO STORAGE)	2.6	-	30.7
EXPERIMENTAL (26 HRS)			
MEM+0.1% BSA - RT	2.6±0.3	100	34.1±1.1

Based on 20,000 counts/individual sample using flow cytometric immunofluorescence

++ ±SEM. Data collected from 4 individual dissociations/ treatment group

+++ RT = room temperature

CS = calf serum

TABLE A2

iGH RELEASED INTO 26 HR STORAGE MEDIUM

TREATMENT	µg GH IN 26 HR STORAGE MEDIUM	ng GH IN STORAGE MEDIUM/1000 GH CELLS/DAY+
MEM+0.1%BSA - RT	23.1±2.2++	33.2±3.2
MEM+0.1%BSA - 4°C	36.1±2.1	46.2±2.7
√MEM+5%CS - RT	21.6±2.7	95.1±12.0
√MEM+5%CS - 4°C	28.8±2.9	35.3±3.5

++ n=4/group. GH levels measured by enzyme immunoassay.

+ Data based on somatotr[illegible]ecovered from each sample.

TABLE A3

CHARACTERISTICS OF GH CELLS AS DETERMINED BY FLOW CYTOMETRY

TREATMENT	PEAK CHANNEL NUMBER FLUORESCENCE+	PEAK CHANNEL NUMBE FALS++
EXP # 1		
CONTROL (NO STORAGE)	25.5±1.5	50.3±7.4
EXPERIMENTAL (26 HRS)		
MEM+0.1%BSA-RT	24.3±0.3	42.3±1.1
MEM+0.1%BSA-4°C	24.8±0.9	41.0±0.7
√MEM+5%CS-RT	27.0±1.2	40.5±1.9
√MEM+5%CS-4°C	25.0±0.7	40.0±0.4
EXP # 2		
CONTROL (NO STORAGE)	26	63
EXPERIMENTAL (26 HRS)		
MEM+0.1%BSA-RT	23.5±0.7	48.8±4.8

+ Value represents intensity of GH-FITC signal at fluorescent peak. Scaling = log. Intensity of signal is proportional to GH content.

++ Value represents forward angle light scatter channel # at peak. Scaling = linear. Magnitude of signal is related to cell size.

TABLE A4

GH RELEASED FROM CELLS IN CULTURE+

TREATMENT	µg iGH IN 3 DAY CULTURE MEDIUM	ng GH/1000GH CELLS SEEDED	ng GH/1000 GH CELLS/ DAY
EXP # 1			
CONTROL (NO STORAGE)	1.25±0.04	68.2±2.4	22.7±0.8
EXPERIMENTAL (26 HRS)			
MEM+0.1%BSA - RT	1.10±0.05	48.9±2.1	16.3±0.7
MEM+0.1%BSA - 4°C	1.01±0.08	48.4±3.1	16.1±1.0
∂MEM+5%CS-RT	1.02±0.08	95.5±7.2	31.8±2.4
∂MEM+5%CS-4°C	1.00±0.13	46.1±5.8	15.4±1.9
EXP # 2			
CONTROL (NO STORAGE)	1.75±0.13	85.4±6.2	28.5±2.1
EXPERIMENTAL (26HRS)			
MEM+0.1%BSA-RT	1.37±0.06	60.1±2.5	20.0±0.8

+ 66,000 cells/well in 250LL _MEM+5%calf serum. Each dissociation sample was seeded into triplicate wells. Thus, culture data shown in this table are averages from 12 determinations/treatment group.

TABLE 1

TEST PROCEDURES AND CELL REQUIREMENTS
FOR 1887 MISSION

PURPOSE	PROCEDURE	APPROXIMATE NUMBER CELLS REQUIRED
Determine % GH and PRL Cells	Flow Cytometry	6×10^5
Image analysis of GH staining	Immunocytochemistry	2×10^5
Determine intracellular GH content	Extraction	6×10^5
Determine GH secretion in vitro (serum and serumless media)	Cell Culture-immunoassay/bioassay	5×10^4/well (8 wells)
Determine GH secretion from individual cells	Cell blotting	6×10^3/blot (5 blots/rat)
Determine GH secretion in vivo	Cell transplantation into hypophysectomized rats	4×10^6
Characterize GH variants secreted in vitro	HPLC	----

TABLE 2

Levels of Immunoactive and Bioactive GH Released into Transport Medium During 30 hrs. Between Time of Pituitary Removal and Tissue Dissociation in Moscow

	iGH			bGH	
SAMPLE	µg/ml	TOTAL µg IN VIAL		µg/ml	TOTAL µg IN VIAL
F-COS	1.95±0.10+	42.9±2.2	F-COS	1.13±010+	24.9±2.2
S-COS	1.55±0.19	34.1±4.2	S-COS	2.88±0.12	61.6±2.6

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+ Values represent averages of hormone released from each of 5 glands into 5 individual vials. iGH, F vs S $p < 0.05$; bGH, F vs S $p < 0.001$

TABLE 3

FLOW CYTOMETRIC ANALYSES OF PITUITARY GH CELLS OBTAINED FROM RATS FLOWN ON COSMOS 1887

PARAMETER	F-COS	S-COS	V-1 COS	V-2 COS
GH				
1). % GH CELLS[1]	26.6	22.9	23.4	24.3
2). BRIGHTNESS FACTOR[2]	24.5X	13.1X	9.2X	___[3]
3). PEAK CHANNEL-FALS[4]	181	151	169	147
4). PEAK CHANNEL-PLS[5]	145	145	147	143

[1] Based on counts of 50,000 - 60,000 cells

[2] Relative to unstained cells; based on marker index; i.e., fluorescence intensity converted to voltage. Ratio of stained to unstained cells = marker index.

[3] Could not be determined accurately since different filter gates were used.

[4] FALS = forward angle light scatter. Peak channel is related to cell size.

[5] PLS = perpendicular light scatter. Peak channel proportional to internal structure ("Granularity").

TABLE 4

Reassay of Selected Samples Obtained from Serum-Containing Cultures

Sample*	Days In Culture	Assay #1** ng iGH released/1000 GH	Assay #2 cells seede
F-COS	3	62±5	77±5
S-COS	3	57±4	55±8
V-1 COS	3	85±8	79±7
V-2 COS	3	96±5	86±5
F-COS	6	73±10	80±9
S-COS	6	143±7	135±9
V-1 COS	6	102±7	88±7
V-2 COS	6	151±7	128±·

* From calf-serum cultures only.

** These data are also plotted in Fig. 6.

TABLE 5

bGH Released in vitro From Pituitary Cells
Prepared From Rats Flown on Cosmos 1887

GROUP	bGH (ug/ml culture media)
F-COS	0
S-COS	2.34 (2.03-2.63)+
V-1	1.94 (1.79-2.06)
V-2	1.44 (1.40-1.57)

+ 95% confidence intervals. See text for description of assay conditions.

TABLE 6

INTRACELLULAR iGH AT THE BEGINNING AND END OF CULTURE

GROUP	INTRACELLULAR iGH AT START OF CULTURE (ng iGH/10^3 GH CELLS)*	INTRACELLULAR iGH AT END OF CULTURE (ng iGH/10^3 GH CELLS)*		iGH SYNTHESIS+ (ng iGH/10^3CELLS)	
		CALF SERUM	DEFINED MEDIUM	CALF SERUM	DEFINED MEDIUM
F-COS++	40±17 (4)	8.6±0.8 (3)	11.9±3 (5)	104	65
S-COS	61±17 (5)	22±2 (5)	13.5±2 (5)	161	78
V-1 COS	133±19 (5)	24±1 (5)	36±4 (5)	77	24
V-2 COS	102±15 (5)	42±2 (5)	23±3 (5)	186	75

+ (GH in culture media + GH in cells at end of culture) - intracellular GH at start of culture.

++F-COS = Flight; S-COS = synchronous control; V-1 COS = 1st vivarium exeriment; V-2 COS = 2nd vivarium experiment

* Expressed on the basis of the numbers of GH cells seeded (determined by flow cytometric immunofluorescence, see Table 3)

TABLE 7

GROWTH HORMONE SECRETION FROM CELLS OF S-COS AND F-COS RATS IMPLANTED INTO CEREBRAL VENTRICLES OF HYPOPHYSECTOMIZED RATS*

TREATMENT GROUPS	TIBIAL WIDTH (μ)	μg bGH+ SECRETED FROM IMPLANT
S-COS EMPTY FIBER	155±0.5 (11	0
S-COS CELL FIBER	177±1.3 (7)	0.22±0.03
F-COS EMPTY FIBER	155±0.3 (10)	0
F-COS CELL FIBER	164±0.6 (10)	0.10±0.01#

* Each hypophysectomized rat was implanted with a hollow fiber containing $2x10^5$cells. Animals were killed 10 days post implantation and fiber placement was verified by visual inspection of sliced brain tissue. Animal receiving fibers which did not come in contact with a ventricular surface were considered unsuccessful and are excluded. The number of rats receiving successful implants are shown in ( ).

+ Estimated from responses of other hypophysectomized rats receiving 4 daily injections of bovine GH standard.

# $p < .05$

TABLE 8

HPLC FRACTIONATION AND GH ASSAY OF 3 AND 6 DAY, SERUMLESS CULTURE MEDIUM FROM F-COS AND S-COS CELLS.

SAMPLE	% OF RECOVERED iGH IN FRACTION		
	FR. 1	FR. 2	FR. 3
F-COS (3 DAY)	8.5	84.5	7.0
S-COS (3 DAY)	11.5	80.8	7.9
F-COS (6 DAY)	2.2	91.4	7.4
S-COS (6 DAY)	2.6	93.8	3.6

+ Fr. 1 = void volume (n62K); Fr. 2 = 18-62K; Fr. 3 = low molecular weight
GH recoveries after HPLC, dialysis, lyophylization and
reconstitution ranged from 21-63%. Results represent average of 2
independant immunoassays for 3 day samples; one for 6 day samples.

TABLE 9

FLOW CYTOMETRIC ANALYSES OF PITUITARY PRL CELLS OBTAINED FROM RATS FLOWN ON COSMOS 1887

PARAMETER	F-COS	S-COS	V-COS
PRL			
1). % GH CELLS[1]	46.7	31.8	35.7
2). BRIGHTNESS FACTOR[2]	4.1X	5.2X	6.3X
3). PEAK CHANNEL-FALS[3]	92	64	108
4). PEAK CHANNEL-PLS[4]	40	24	32

1 Based on counts of 50,000 - 60,000 cells

2 Could not be determined accurately since different filter gates were used.

3 FALS = forward angle light scatter. Peak channel is related to cell size.

4 PLS = perpendicular light scatter. Peak channel proportional to internal structure ("Granularity").

TABLE 10

INTRACELLULAR PRL AT THE BEGINNING AND END OF CULTURE

GROUP	INTRACELLULAR PRL AT START OF CULTURE (ng PRL/10s PRL CELLS)*	INTRACELLULAR PRL AT END OF CULTURE (ng PRL/10s PRL CELLS)*	
		CALF SERUM	DEFINED MEDIUM
F-COS	0.22±0.05	4.6±0.6	1.4±0.3
S-COS	0.24±0.02	4.9±0.4	1.4±0.3
V-COS	0.26±0.06	7.6±0.7	2.2±0.2

* Expressed on the basis of the numbers of GH cells seeded (determined by flow cytometric immunofluorescence)

TABLE 11

COMPARISON OF GH CELL RESULT BETWEEN
THE COSMOS 1887 AND SL-3 MISSIONS

TEST	COSMOS FLIGHT/CONTROL	SL-3* FLIGHT/CONTI
1) iGH released into storage medium before cell preparation	↑1.25	ND[1]
bGH released into storage medium before cell preparaton	↓0.39	ND
2) % GH cells	→1.0	→1.0
3) GH fluorescence staining	↑2.0	↑1.16
4) GH cell size (FALS)	↑1.2	↑1.05
5) GH cell cytoplasmic "Granularity"	→1.0	ND[2]
6) Short term (2 hr.) GH secretion assay (cell blot)	↑1.19	ND[3]
7) 6 day cell culture (iGH)		
3 day medium (CS)	↑1.10	↓0.44
3 day medium (DM)	↑1.24	↓0.83
Next 3 day medium (CS)	↓0.51	↓0.77
Next 3 day medium (DM)	↓0.57	ND[2]
8) 3 & 6 day cell culture medium (bGH)	↓<0.01	ND
9) iGH synthesis in 6 day culture		
(CS)	↓0.64	↓0.39
(DM)	↓0.83	ND[2]
10) Tibial line response after hollow fiber transplantation (in vivo culture; bGH).	↓0.43	↓0.60

* Small rats (200 gm)
↑ = increase; ↓ = decrease
→ = no change.
ND = not done
1 Storage of pituitary tissue not required in this experiment.
2 Insufficient sample for analysis
3 Technique not developed at the time of experiment.

Fig. 1. Photomicrographs of F-Cos and S-Cos cells stained for specific GH immunofluorescence (green). Top panel, F-Cos cells showing red nuclear fluorescence due to propidium iodide. These cells, incubated with monkey non-immune serum, do not show cytoplasmic staining. Middle panel, S-Cos cells. Bottom panel, F-Cos cells. Note obvious brighter green fluorescence in F-Cos cells.

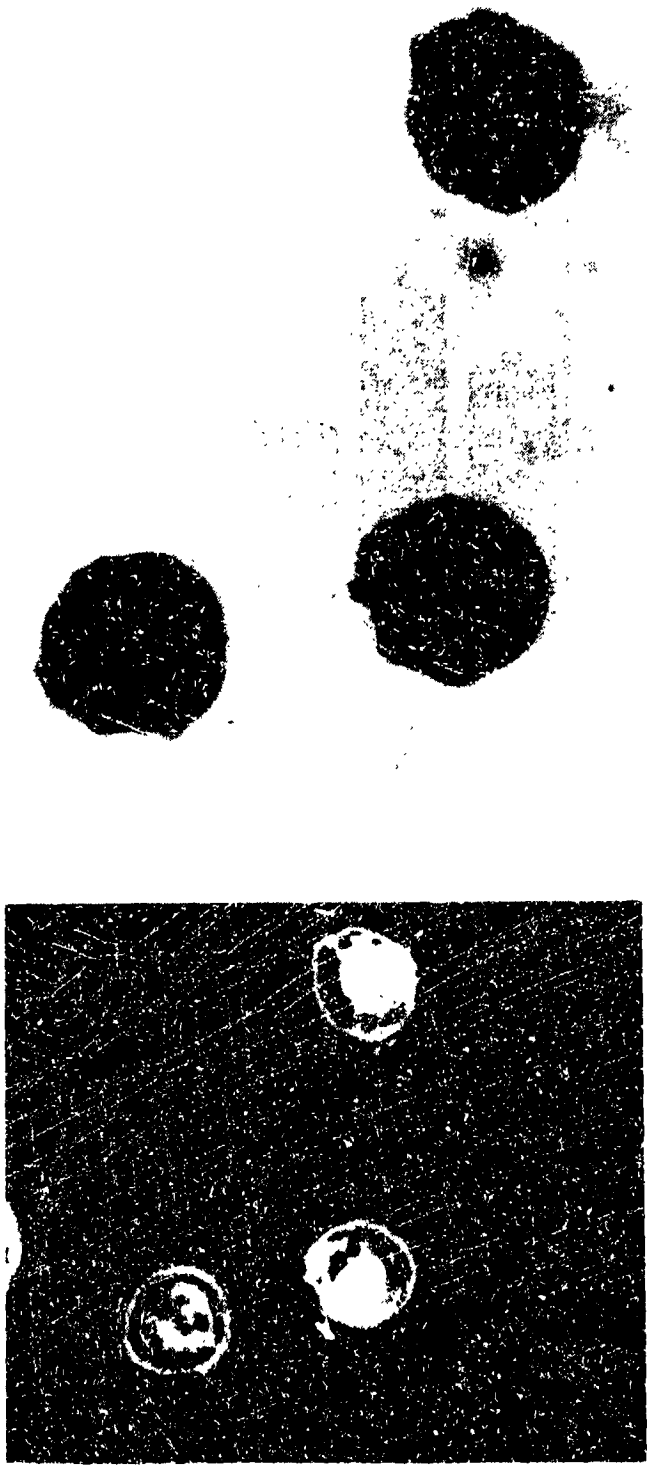

Fig. 2 (top). GH cells immunocytochemically stained for GH. The dark reaction product represents GH. Fig. 2 (bottom). Image analysis of same cells as in top figure. This represents a computerized video enhancement image whereby the GH staining in red is color thresholded in a different gray level than the yellow nuclear area. This procedure was done on 100 cells in each group to yield the information shown in Fig. 3.

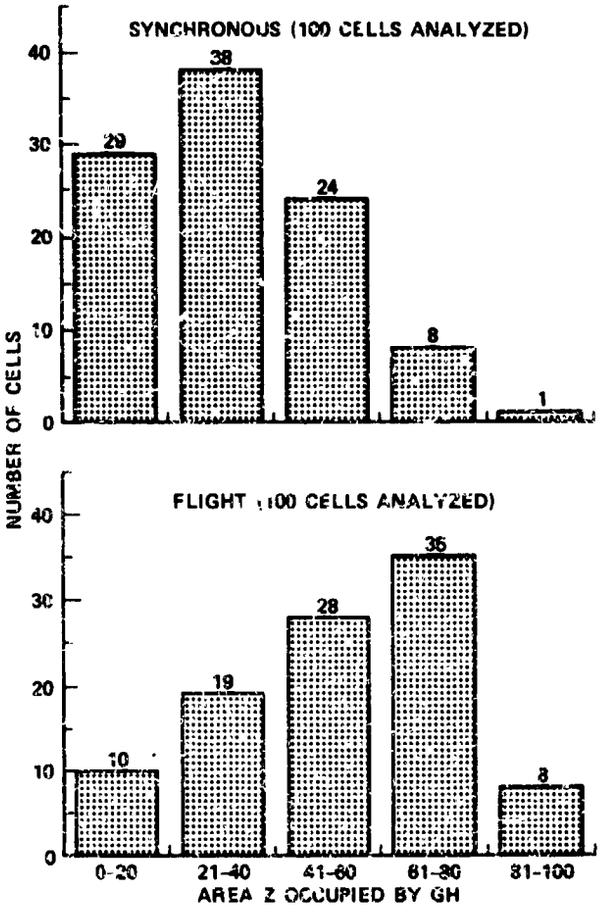

Fig. 3. Image analysis of 100 cells in both F-Cos and S-Cos groups done according to procedure shown in Fig. 2.

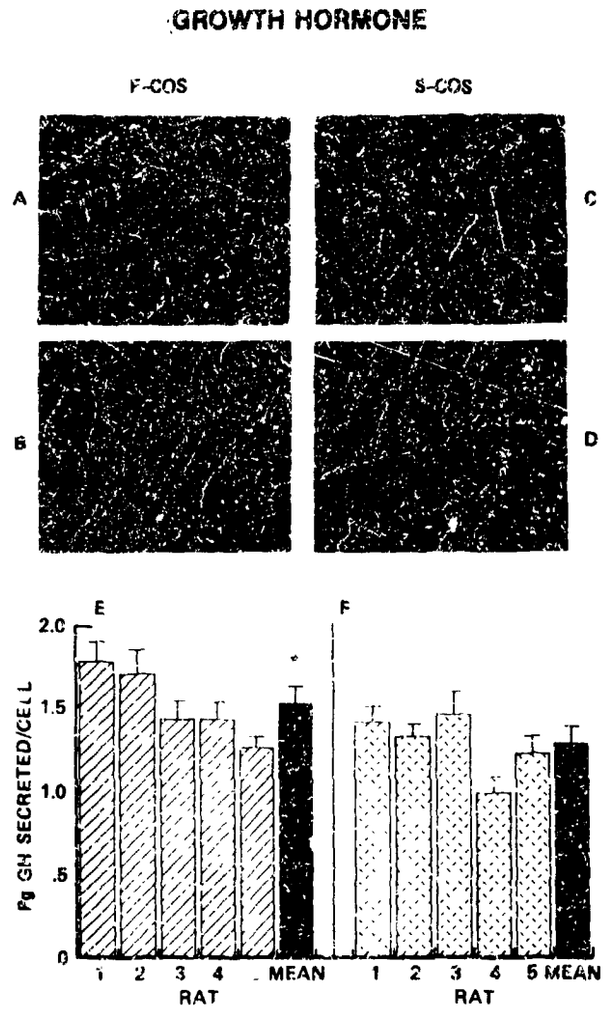

Fig. 4. Top: GH cell blot images produced by individual cells from S-Cos and F-Cos groups. Panel A: F-Cos cell blot. Panel B: video enhanced image of same F-Cos cells ( ). Panel C: S-Cos cell blot. Panel D: video enhanced image of same S-Cos cells. Panel E and F: quantitation of GH released from 35 cells/rat/group. The mean GH secretion of all cells (175) in each group is shown in black bar. * F-Cos secretion, $p < 0.05$ vs. S-Cos cells.

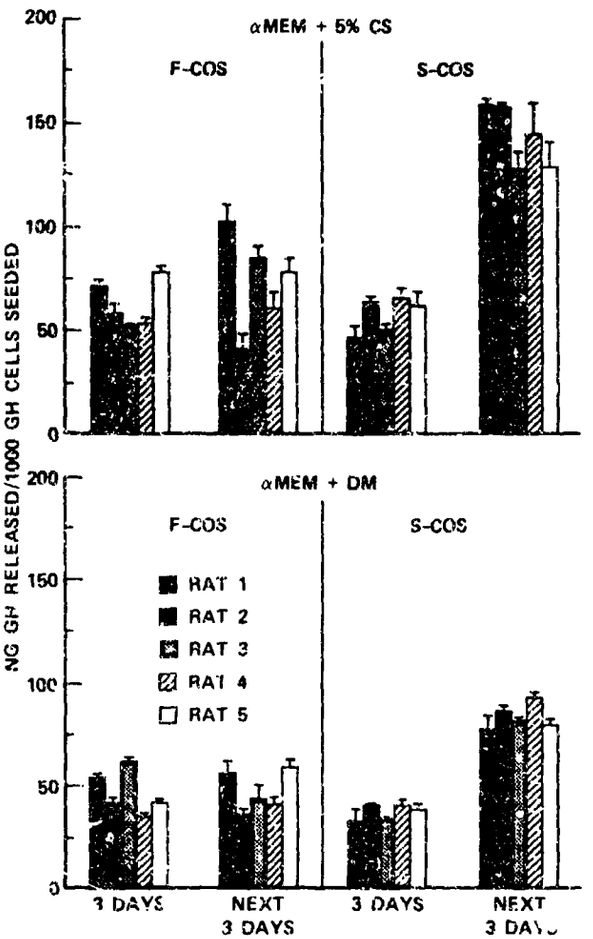

Fig. 5. Top Panels: GH released from pituitary cells of individual rats in both F-Cos and S-Cos groups. Serum containing medium. Error bars represent sem's of GH from 4 culture wells. Bottom Panels: GH released from pituitary cells of individual rats in both F-Cos and S-Cos groups. Serumless medium.

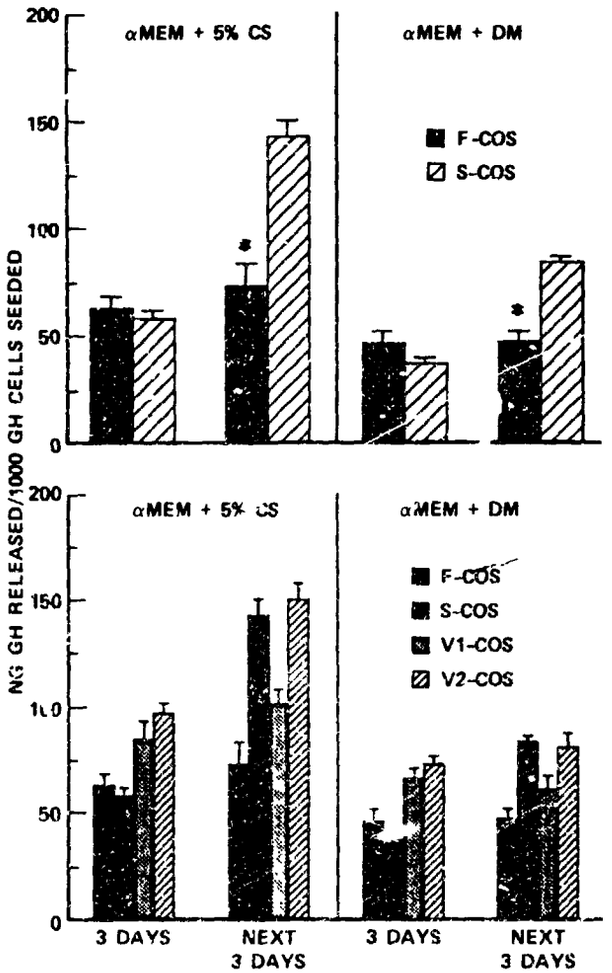

Fig. 6. Top Panels: Combined GH secretion data from cells of individual rats (Fig. 5). Bottom panels: Comparison of GH secretion from combined cells of [illegible] treatment groups.

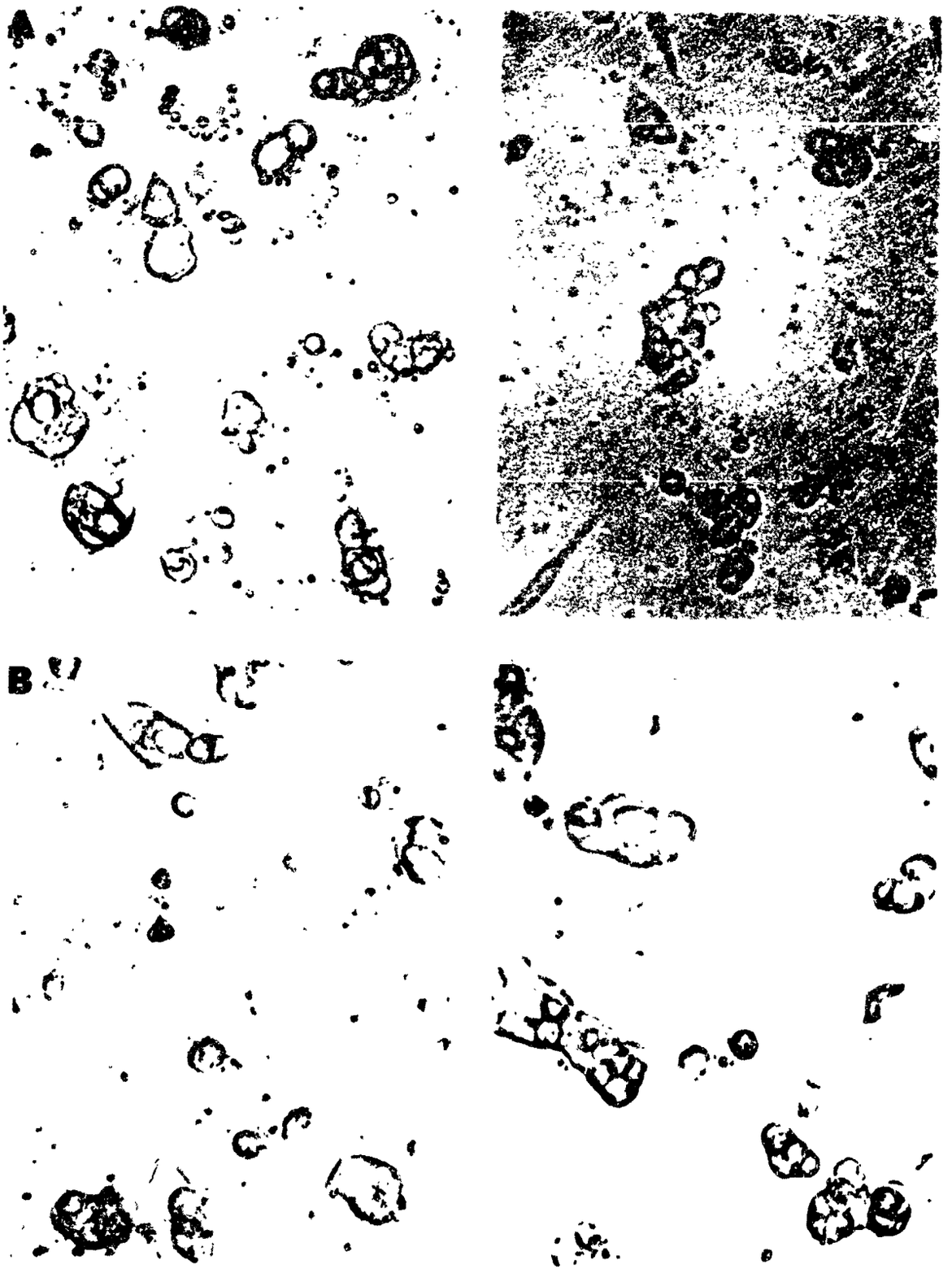

Fig. 7A. Photomicrographs of pituitary cells from S-Cos and F-Cos animals in culture for 3 and 6 days in serum containing medium. A) S-Cos, 3 day; B) S-Cos, 6 day; C) F-Cos, 3 day; D) F-Cos, 6 day.

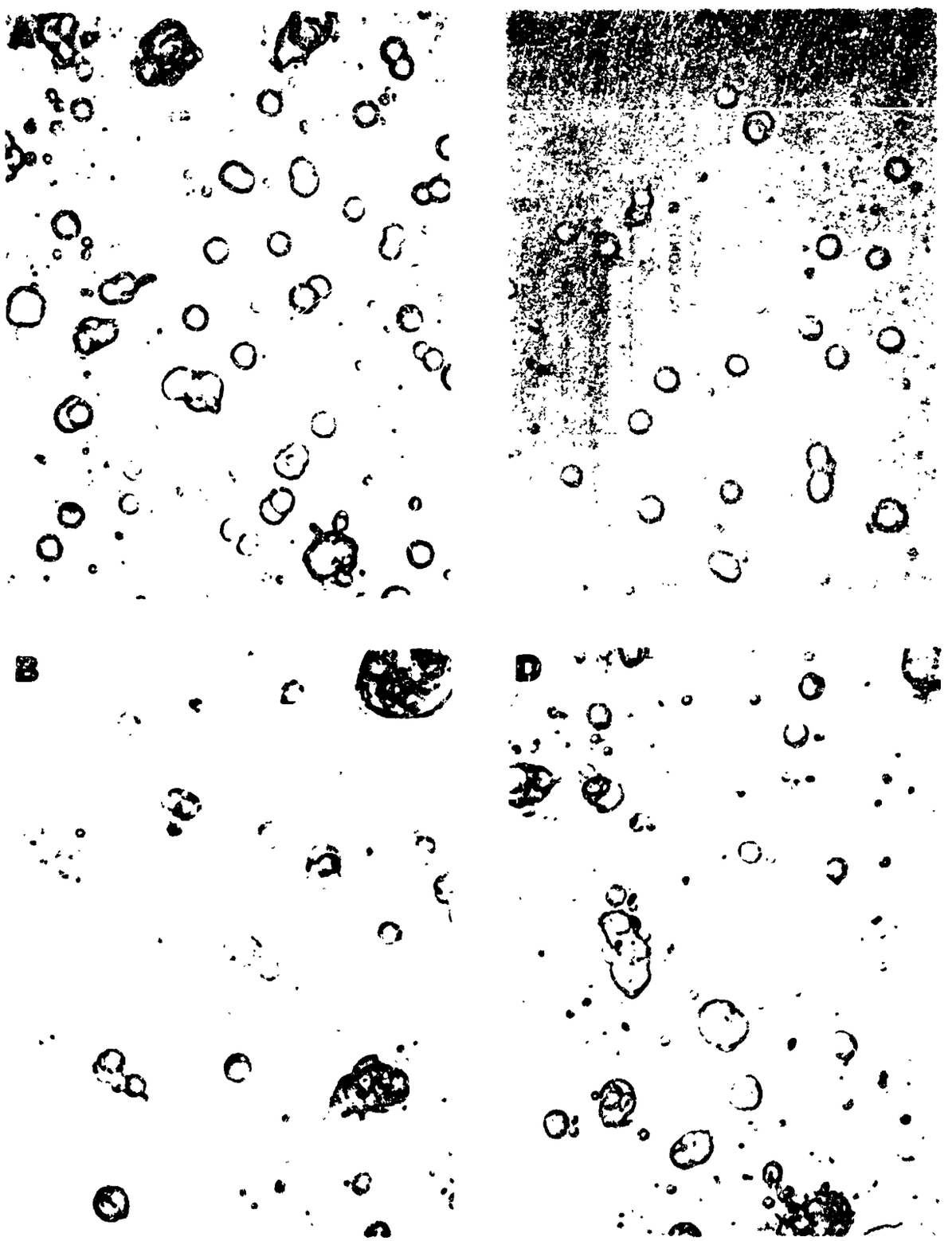

Fig. 7B. Photomicrographs of pituitary cells from S-Cos and F-Cos animals in culture for 3 and 6 days in serum free medium. A) S-Cos, 3 day; B) S-Cos, 6 day; C) F-Cos, 3 day; D) F-Cos, 6 day.

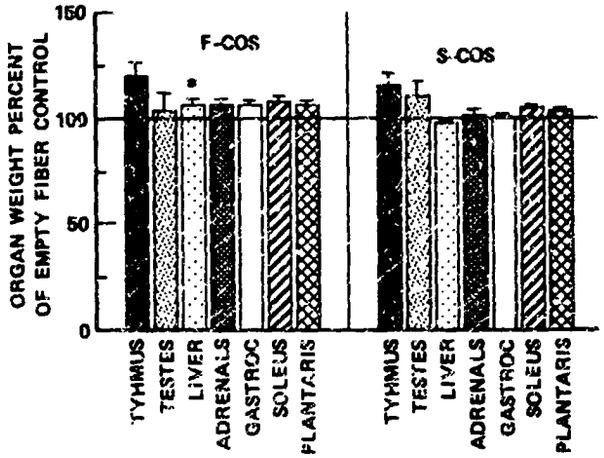

Fig. 8. Organ weights of hollow fiber implant recipients expressed as a percentage of the empty fiber controls. Actual organ weights of animals receiving empty hollow fibers shown in table.

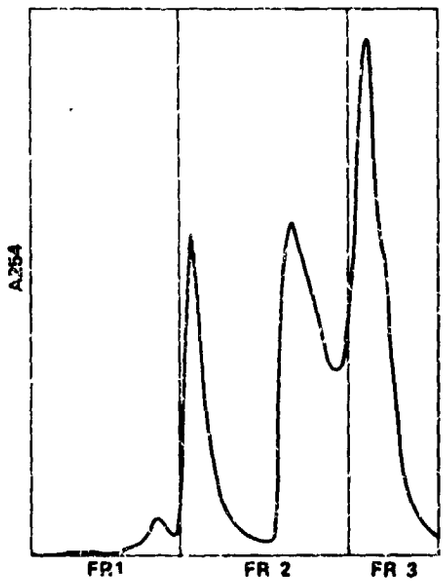

Fig. 9. Optical density profile (A254 nm) of proteins contained in 3 day culture medium from F-Cos cells. The GH contents contained in fractions 1-3 are shown in table 4.

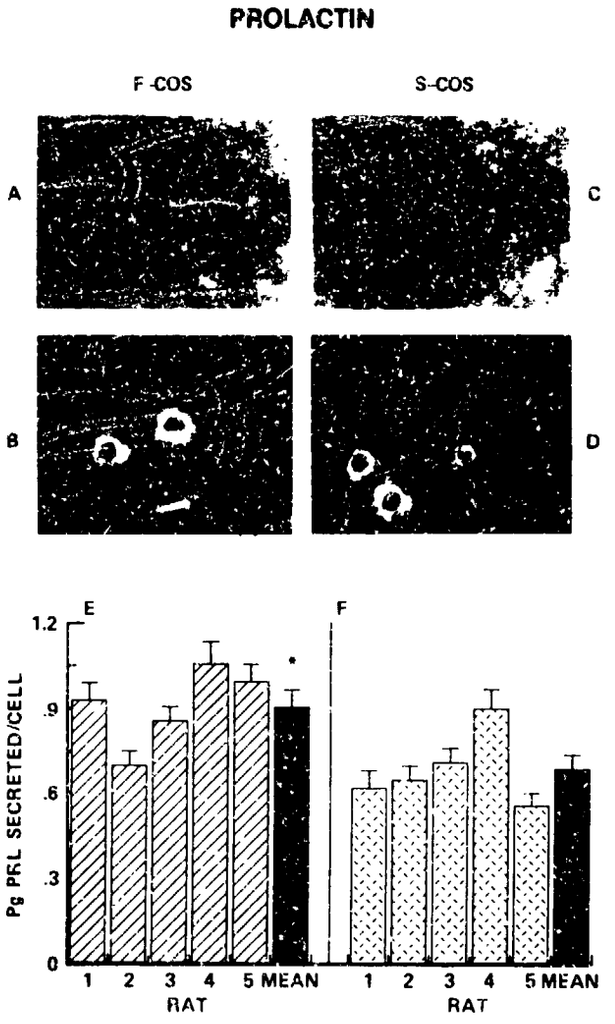

Fig. 10. Top: PRL cell blot images produced by individual cells from S-Cos and F-Cos groups. Panel A: F-Cos cell blot. Panel B: video enhanced image of same F-Cos cells ( ). Panel C: S-Cos cell blot. Panel D: video enhanced image of same S-Cos cells. Panel E and F: quantitation of PRL released from 35 cells/rat/group. The mean PRL secretion of all cells (175) in each group is shown in black bar. * F-Cos secretion, $p < 0.05$ vs. S-Cos cells.

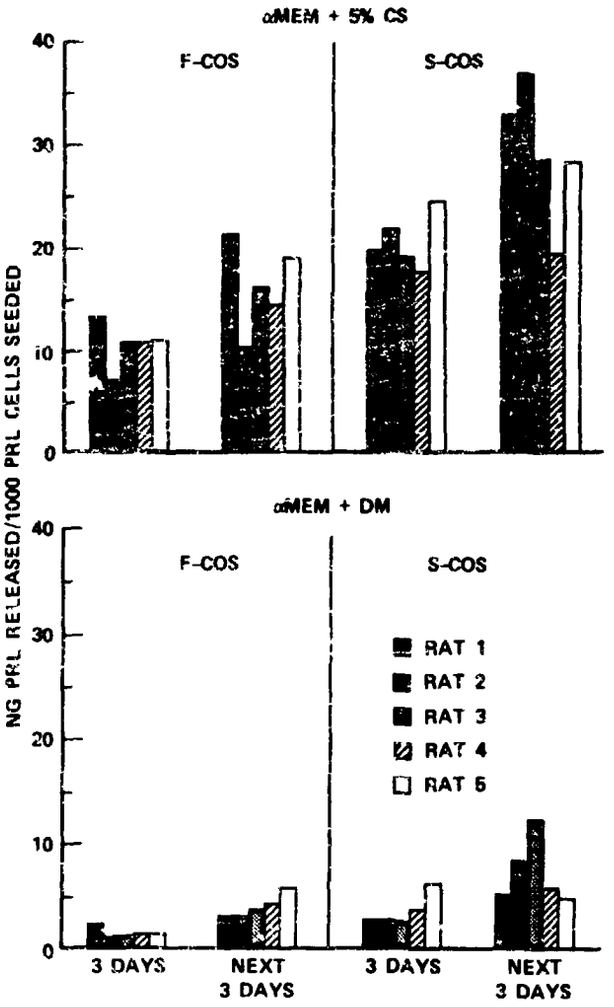

Fig. 11. Top Panels: PRL released from pituitary cells of individual rats in both F-Cos and S-Cos groups. Serum containing medium. Bottom Panels: PRL released from pituitary cells of individual rats in both F-Cos and S-Cos groups. Serumless medium.

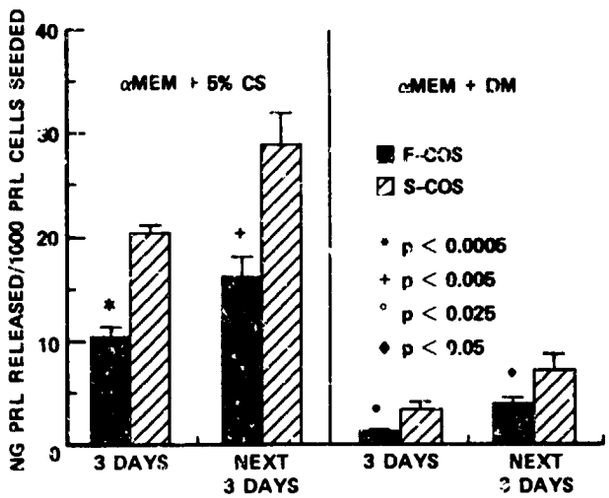

Fig. 12. Combined PRL secretion data from cells of individual rats (Fig. 11).

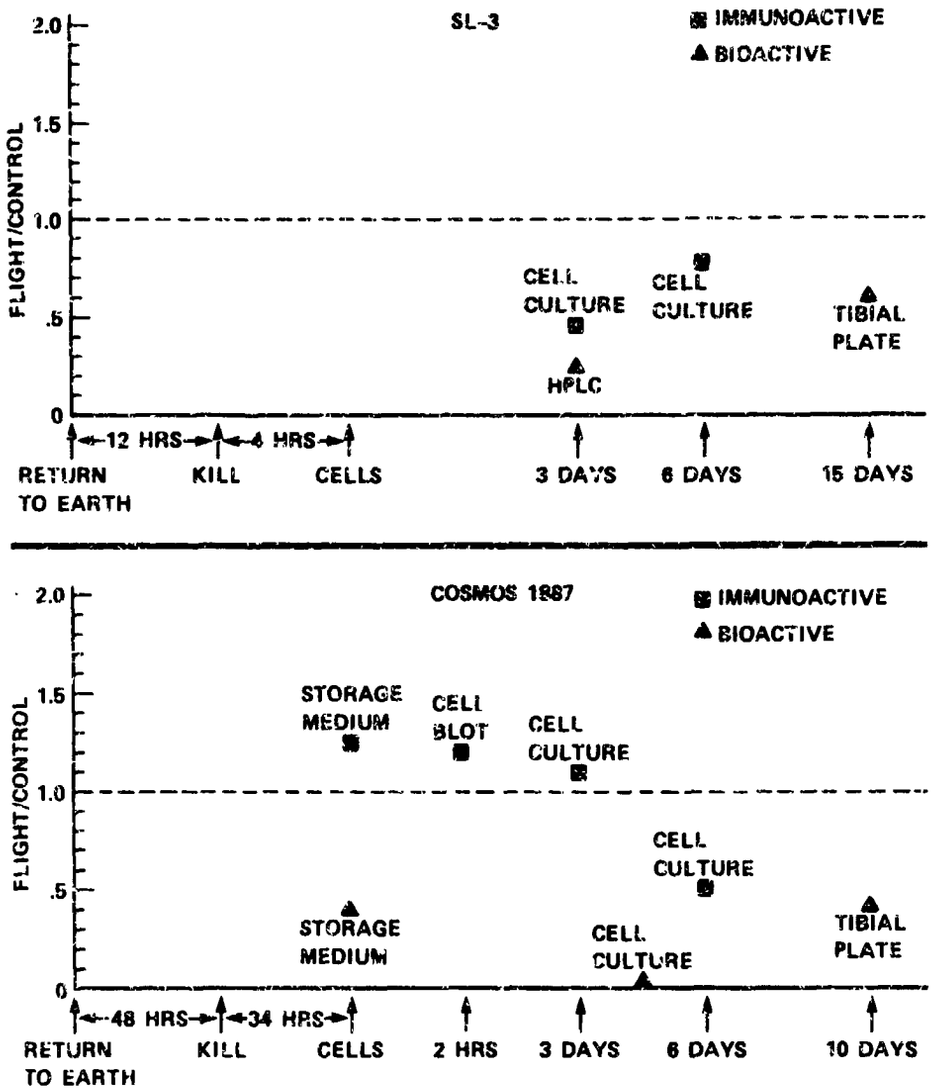

Fig. 13. Effect of spaceflight on subsequent release of bioactive and immunoactive GH release in vivo and in vitro: Comparison of the SL-3 and 1887 experiments.

EXPERIMENT K-6-22

PART II: IMMUNOHISTOCHEMICAL ANALYSIS OF HYPOTHALAMIC HORMONES

W. Vale, P. Sawchenko, and I. Krasnov

SUMMARY

It was originally anticipated that blocks of hypothalamic tissue would be prepared for radioimmunoassay of hypophysiotropic hormones mediating somatic growth (growth hormone-releasing factor, somatostatin), and stress-related corticotropin secretion (corticotropin-releasing factor). Each of these peptides is expressed in hypothalamic neurons that project directly to the hypophyseal portal vasculature for delivery to the anterior lobe of the pituitary. Even within the hypothalamus, however, each is also rather broadly distributed in cell bodies and/or axons that bear no ostensible relationship to their hypophysiotropic functions. Because of this, it was decided to attempt to employ immunohistochemical methods in an attempt to better localize any effects of flight on these neuropeptide systems.

MATERIALS AND METHODS

Blocks of fresh-frozen hypothalamic tissue were stored upon receipt at -70 deg. C. The fixation protocol employed was based on preliminary studies in which we attempted to maximize antigenicity and morphologic preservation in fresh frozen samples. The tissue was transferred for 30 minutes to a -20 deg. C freezer and then for 10 minutes to a refrigerator at 4 deg. C. The blocks were fixed for 6 hours in ice cold 4% paraformaldehyde in 0.1 M phosphate buffer, and then overnight at 4 deg. C in the same fixative containing 10% sucrose. The following day, the blocks were frozen in dry ice and five 1-in-5 series of 20 μm thick sections were cut on a sliding microtome. Sections were rinsed (2 x 10 minutes) in 0.05 M phosphate-buffered saline (PBS) and stored at -20 deg. C in cryoprotectant (30% ethylene glycol, 15% sucrose in 0.05 M phosphate buffer) until staining.

A conventional indirect immunofluorescence method (Gerfen and Sawchenko, 1984) was used for staining. Complete series of sections through the hypothalamus of each member of the flight and synchronous control groups were incubated in primary antisera raised in rabbits against rat hypothalamic growth hormone-releasing factor (GRF; serum G75 of Sawchenko, et al., 1985), somatostatin-28 (SS-28; serum S309 of Benoit, et al., 1982; see also Sawchenko, et al., 1988), rat corticotropin-releasing factor (CRF; serum C70; see Sawchenko, 1987) and arginine vasopressin (AVP; Vandesande and Dierickx, 1975; see Sawchenko, et al., 1984). Details of the characterization of these sera for immunohistochemical studies may be found in these references. All primary antisera were localized with an affinity purified, fluorescein-conjugated, goat anti-rabbit IgG (Tago, Inc., Burlingame, California).

RESULTS

Positive staining patterns, consistent with their acknowledged distributions, were obtained for each of the four peptides examined. Despite the fact that the immersion fixation protocol employed permitted localization, staining was less robust and, in the case of the median eminence, less discretely localized, than that obtainable in perfusion-fixed rats of comparable age.

Somatostatin-28. SS-28-immunoreactive (IR) cell bodies were found consistently in members of both groups in the anterior periventricular nucleus of the hypothalamus, the acknowledged primary source of hypophysiotropic SS-IR pathways. Correspondingly, immunoreactive fibers were seen in the

external lamina of the median eminence, though resolution of these was poor (Figure 1). Other acknowledged SS-IR terminal fields, for example in the ventromedial and paraventricular nuclei of the hypothalamus were moderately well-stained. Comparison of the staining patterns between the two groups showed the flight animals to be consistently less robust. This was manifest primarily in the forms of a lesser number of immunostained cell bodies in the anterior periventricular region, and a lesser intensity of staining of fibers in each of the regions enumerated above (see Figures 1 and 2).

Growth Hormone-Releasing Factor. GRF-IR appeared as a relatively diffuse band spanning the external lamina of the median eminence, and as isolated, and more discretely stained, fibers in hypothalamic regions (e.g., dorsomedial, paraventricular, anterior periventricular nuclei) shown previously to receive GRF-IR inputs (Sawchenko, et al., 1985). Perikaryal staining, which normally requires pretreatment with colchicine, was not observed. As was the case with SS-IR, staining for GRF-IR in the median eminence appeared consistently less intense in the flight animals, relative to synchronous controls (Figure 1).

Arginine Vasopressin. (AVP-IR was present in both groups in cell bodies of the paraventricular, supraoptic and suprachiasmatic nuclei, and in the hypothalamo-hypophyseal tract, coursing through the hypothalamus and the internal lamina of the median eminence. No consistent differences in staining between the two groups were encountered for this antigen. Perikaryal staining for AVP-IR in rats of both groups was somewhat unusual and worthy of comment. Staining in both magnocellular neurosecretory cell bodies was weak and granular in appearance, while staining of dendritic processes was robust. By contrast, staining of cells in the suprachiasmatic nuclei was unremarkable. No Herring Body-like swellings, which might be indicative of acute dehydration, were observed on magnocellular AVP-IR fibers.

Corticotropin-Releasing Factor. As was the case for GRF, staining for CRF-IR was weak and limited the median eminence and isolated intrahypothalamic fibers. No consistent differences in staining of either fiber type between groups was evident.

DISCUSSION

In summary, we observed consistently lesser staining intensities for both SS-28- and GRF-IR in flight, relative to synchronous control groups, and no such alterations in staining for CRF or AVP. AVP-IR in magnocellular neurosecretory perikarya appeared unusually weak in members of both groups. The fact that staining for both of the principles involved most directly in the central regulation of growth hormone secretion appeared to be affected at least somewhat selectively may be viewed as suggesting a specific neuroendocrine dysfunction within the central nervous system, some caution is warranted. The sub-optimal fixation protocol, and the (presumably associated) diffuse staining of fibers and terminals in the median eminence, must temper any interpretation of the data. Moreover, the fact that one of the ostensibly unaffected principles, AVP, is normally expressed at concentrations in hypothalamic perikarya that saturate our detection system, would lead one to question the capacity of this methodology to resolve subtle differences. Finally, the fact that both the stimulatory and inhibitory principles appeared driven in the same direction is perplexing. This could represent a compensatory or counterregulatory response of one system to a perturbation in the other. The present findings provide no insight into this issue.

REFERENCES

1. Benoit, R., Line, N., Alford, B., and Guillemin, R. (1982). Seven peptides derived from pro-somatostatin in rat brain. Biochem. Biophys. Res. Commun., 107:944-950.

2. Gerfen, C.R., and Sawchenko, P.E. (1984). An anterograde neuroanatomical tracing method that shows the detailed morphology of neurons, their axons and terminals: Immunohistochemical localization of an axonally transported plant lectin, Phaseolus vulgaris - leucoagglutinin. Brain Res., 290:219-238.

3. Sawchenko, P.E. (1987). Evidence for differential regulation of CRF- and vasopressin-immunoreactivities in parvocellular neurosecretory and autonomic-related projections of the paraventricular nucleus. Brain Res. 437:253-263.

4. Sawchenko, P.E., Benoit, R., and Brown, M.R. (1988). Somatostatin 28-immunoreactive inputs to the paraventricular nucleus: Origin from non-aminergic neurons in the nucleus of the solitary tract. J. Chem. Neuroanat. 1:81-94.

5. Sawchenko, P.E., Swanson, L.W., Rivier, J. and Vale, W.W. (1985). The distribution of growth hormone-releasing factor (GRF) -immunoreactivity in the central nervous system of the rat: An immunohistochemical study using antisera directed against rat hypothalamic GRF. J. Comp. Neurol., 237:100-115.

6. Sawchenko, P.E., Swanson, L.W., and Vale, W.W. (1984). Corticotropin-releasing factor: Co-expression within distinct subsets of oxytocin-, vasopressin-, and neurotensin-immunoreactive neurons in the hypothalamus of the male rat. J. Neurosci., 4:1118-1129.

7. Vandesande, F., Dierickx, K., and DeMey, J. (1975). Identification of separate vasopressin-neurophysin II and oxytocin-neurophysin I containing nerve terminals in the external region of the bovine median eminence. Cell Tiss. Res., 158:509-516.

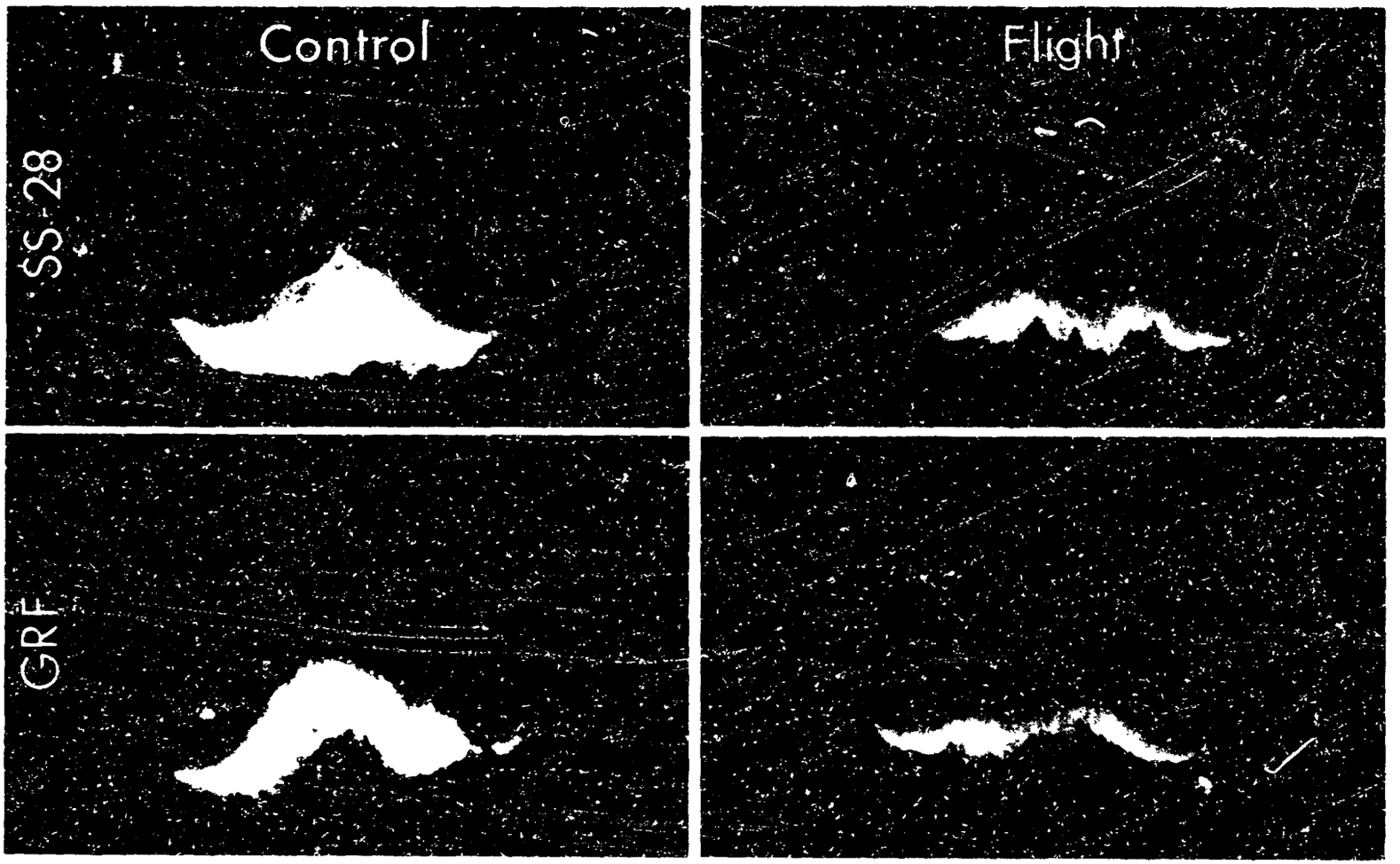

Figure 1. Fluorescence photomicrographs of frontal sections through the medial basal hypothalamus of representative rats from the synchronous control and flight groups, stained immunohistochemically for SS-28 (top) or GRF. For both peptides, staining seen in the external lamina of the median eminence was less intense in the flight group. The third ventricle appears near the center of each micrograph. All micrographs X 75.

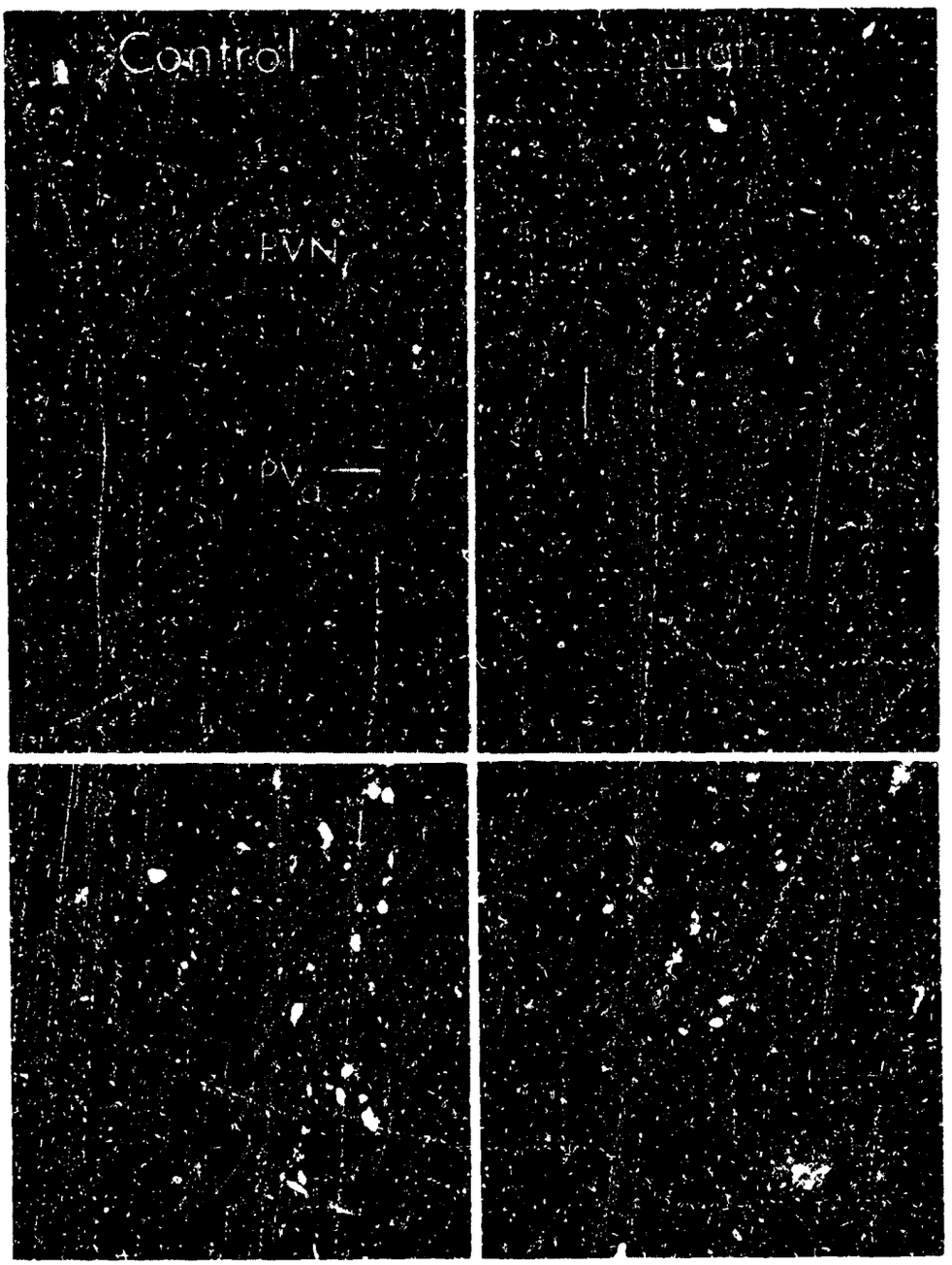

Figure 2. Fluorescence photomicrographs of frontal sections through the anterior hypothalamus to show SS-28-IR perikarya and fibers in representative animals from the flight and synchronous control groups. In the anterior periventricular nucleus (PV_a) fewer, and less intensely stained perikarya are seen in the section from the flight animal. Top two micrographs X 50. The bottom panel shows a higher magnification (X 150) view through the paraventricular nucleus (PVN). Note the pronounced difference in the number of stained fibers.

EXPERIMENT K-6-22

PART III: PLASMA ANALYSIS

R. Grindeland, I. Popova, and M. Vasques

INTRODUCTION

Plasma hormone and biochemical analyses were performed either in our laboratory or by a clinical laboratory. Results of these tests were made available to all U.S. investigators to facilitate their evaluation of animal physiological status and interpretation of their data. Specific measurements are discussed in the context of the relevant experiments. For example, phosphorus, calcium, and alkaline phosphatese values are considered with bone studies, plasma proteins and albumin concentrations are discussed with the liver enzyme studies, and testosterone titers are discussed with the testes and pineal gland investigations.

PROCEDURES

Trunk blood was collected after decapitation into tubes containing 50 ml ammonium heparin. Blood biochemical measurements were determined in automated analysis (SMAC) by Veterinary Reference Laboratory, San Leandro, CA. Plasma immunoactive growth hormone was determined in- house by an adaptation of the radioimmunoassay of Schalch and Reichlin (1). Interassay variation was 6% and intraassay variation 3.8%. Rat growth hormone standard (3 USP U/μg) and antisera, raised in monkeys, were produced in-house. The prolaction radioimmunoassay procedure was also an adaptation of the growth hormone RIA method(2). Rat prolactin (29U/μg) and rabbit antisera were produced in our laboratory. Inter and intra assay variation were 4% and 2% respectively. Testosterone and corticosterone were assayed using immunoassay kits. The testosterone kit was obtained from Diagnostic Products Corp., Los Angeles, CA and the Corticosterone kit from Radioassay Laboratories, Inc., Carson, CA. Sensitivities for these assays are 0.05-0.01 ng/ml of the possible circulating steriods in the male rat. The corticosterone antibody cross reacts with cortisol less than 0.2%; all other steriods cross react less than 0.1%. The testosterone antibody cross reacts with two other androgenic steriods as follows: androstendione-3%; 5-alpha androstan-3 beta, 17 beta-diol-0.5%, and 5- androsten3-beta; 17-beta diol-0.7%. All other physiological steriods cross react less than 0.1%.

RESULTS AND DISCUSSION

Plasma glucose was significantly elevated in Flight (F) compared to Synchronous Control (S) rats, (Table 1). These two groups, fed a half day's portion of food some 20 hours before sacrifice, were most probably fasted. F glucose concentrations were higher than Vivarium Controls (V) but the same as Basal Controls (B) levels. The fasting state of V and B rats is uncertain, so the significance of the glucose levels in the later group is open to question. The elevated glucose levels in F rats, similar to that observed in Space Laboratory 3 animals post flight (3), are not attributable to elevated corticosterone or growth hormone titers (see under hormones). Elevated catecholamine titers, especially in response to reentry, are a plausible explanation. However, the markedly increased liver glycogen (K6-14) is consistent with an inflight elevation of plasma glucose. The increased plasma glucose concentration appears to be a response to microgravity but the mechanism remains unclear.

Plasma calcium was lower in F than in V or B rats, but not different from S rats, suggesting a dietary regimen or caging effect (Table 1). In contrast, phosphorus concentrations were higher in F than S

animals, similar to those of V rats and less than those of B animals. Alkaline phosphatase values were 50% higher in the F animals than the S controls, consistant with changes in bone and mineral metabolism discussed elsewhere. The alkaline phosphatase levels in B rats were 2-4 times those of other groups, perhaps reflecting a more rapid bone turnover. The large standard error, however, suggests extensive variability in the B rats.

Plasma sodium concentrations were similar for all groups of rats. Potassium concentrations of F rats were similar to those of S and V animals but less than B controls. The reasons for the higher levels in B rats is not apparent.

Total protein and albumin concentrations were similar for all groups, suggesting no effect of weightlessness. If hemoconcentration occurred in F rats any decrease in protein could be obscured by the loss of plasma volume. The elevated blood urea nitrogen suggests that protein catabolism has been increased in F rats. Moreover, the apparently constant plasma protein concentrations, increased liver weights, and increased creatine levels of F rats suggest that the protein being catabolized is from skeletal muscle.

Plasma immunoreactive growth hormone measurements were statistically similar for all groups of rats (Table 3). Two groups (B,F) each contained one rat with a high value, raising the means and increasing the standard errors appreciably. Deletion of these values brought the means to similar levels, and reduced the standard error, but did not change the statistical significance between groups. Analogous results were observed in Space Laboratory 3 rats (4). However, secretion of bioassayable GH (part 1 of this report; 3) was markedly decreased after flight.

Prolactin concentrations, which ranged from 2.5 to 7 ng/ml, did not differ significantly between any of the groups. ($p>0.05$)

Corticosterone levels did not differ between F and S groups. The S rats, however, had significantly lower levels than either B or V groups, being about one-half as much. The concentrations found in S and F rats were similar to those in flight and control groups on SL-3 (4). The values are all higher than 3-5 µg/dl found in our laboratory for rats which have been handled extensively. It is of interest that adrenals of F rats were hypertrophied but their corticosterone secretion was virtually identical to that of S animals. The data suggest that either there was no lasting effects of weightlessness on adrenal cortical function or that post flight handling obscured any small difference.

Flight rats had decreased levels of testosterone compared to S controls, but similar concentrations to V rats. B rats had higher testosterone concentrations than any other group for unknown reasons. Space Laboratory-3 F rats also had lower testosterone than S controls, but levels similar to those of B and V rats (4). Data from the two flights suggest that the caging of F and S rats may have evoked an increase in testosterone which was supressed in F rats by microgravity and/or reentry. It is not clear whether the decreased testosterone concentrations reflect decreased gonadotropin secretion or decreased Leydig cell function.

REFERENCES

1. Schalch, D.S. and S. Reichlin. Plasma growth hormone concentrations in the rat determined by radioimmunoassay-Influence of sex, pregnancy, lactation, and anasthesia, hypophysectomy and extra sellar piyuitary transplants. Endocrinology 79: 275-280, 1966.

2. Ellis, S. R.E. Crindeland, J.M. Nuenke, and P.X. Callahan. Purification and properties of rat prolactin. Endocrinology 88: 886, 1936.

3. Grindeland, R., W.C. Hymer, M. Farrington, T. Fast, C. Hayes, K. Motter, L. Patil, and M. Vasques. Changes in pituitary growth hormone cells prepared from rats flown on Spacelab 3. American Journal of Physiology 252:R209-R215, 1987.

4. Vasques, M., R.E. Grindeland, M. Martinelli and R. Furlanetto. Effects of 7 days of microgravity on plasma hormone levels. Proceedings of the Fall Meeting of American Physiology Society-International Union of Physiological Sciences, Montreal, 1988.

TABLE 1

BLOOD BIOCHEMISTRY

GROUP	PHOSPHORUS (mg/dl)	CALCIUM (mg/dl)	SODIUM (mg/dl)	POTASSIUM (meq/l)
Flight				
Mean	6.5[1,3]	9.6 [2,3]	142.0	5.08[3]
S.E.	0.17	0.28	2.37	0.21
Synchronous				
Mean	5.8[2,3]	10.1[2]	142.8	4.8[2,3]
S.E.	0.15	0.15	1.02	0.12
Vivarium				
Mean	6.6[3]	10.5	145.2	5.6
S.E.	0.18	0.12	2.42	0.07
Basal				
Mean	7.2	10.4	142.0	5.7
S.E.	0.14	0.07	0.63	0.19

SUPERSCRIPT KEY
$p < 0.05$

1 differs from synchronous.
2 differs from vivarium.
3 differs from basal.

TABLE 1, CONTINUED

BLOOD BIOCHEMISTRY

GROUP	CREATINE (mg/dl)	ALKALINE PHOS (iu/l)	GLUCOSE (mg/dl)	TOTAL PROTEIN (gm/dl)	ALBUMIN (gm/dl)	BUN (mg/dl)
Flight						
Mean	0.60[1,2,3]	236[1,2,3]	155[1,2]	5.64	3.56	33 [1,2,3]
S.E.	0.00	20	8	0.20	0.12	2.6
Synchronous						
Mean	0.40	154[2,3]	126[3]	5.64	3.48	12[2,3]
S.E.	0.06	9	2	0.15	0.08	0.6
Vivarium						
Mean	0.48	108[3]	112[3]	5.52	3.56	16[3]
S.E	0.05	9	7	0.08	0.08	0.4
Basal						
Mean	0.44	418	156	5.36	3.56	15
S.E	0.04	72	2.64	0.04	0.04	1.0

SUPERSCRIPT KEY

$p < 0.05$

1 differs from synchronous.
2 differs from vivarium.
3 differs from basal.

TABLE 2

PLASMA HORMONE CONCENTRATIONS

BASAL	SYNCHRONOUS	FLIGHT	VIVARIUM
IMMUNOREACTIVE GROWTH HORMONE (ng/ml)			
25.4±8.8 (5)* 16.9±2.3 (4)	18.2±3.1 (5)	34.8±18.8 (5)* 16.0±1.4 (4)	13.5±1.0 (5)
No significant differences between groups ($p > 0.05$) * high value included			
PROLACTIN (ng/ml)			
6.7±1.8	2.7±0.5	5.9±1.9	2.6±1.8
No significant differences between groups ($p > 0.05$)			
CORTICOSTERONE (ng/ml)			
26.2±5.1	13.0±1.1	15.1±4.2	27.1±4.5
Synchronous values are significantly lower than Basal or Vivarium ($p < 0.05$)			
PLASMA TESTOSTERONE (mug/ml)			
3.3±0.49	1.52±0.54	0.34±0.12	0.97±0.42
Basal values are significantly greater than for all other groups; Flight values are lower than Synchronous ($p < 0.05$)			