Feeding frequency affects cultured rat pituitary cells in low gravity[1]

W.C. Hymer[a,*], R.E. Grindeland[b], T. Salada[a], R. Cenci[a], K. Krishnan[a], C. Mukai[c], S. Nagaoka[c]

[a]Department of Biochemistry and Molecular Biology. The Pennsylvania State University. University Park. PA 16802. USA
[b]NASA Ames Research Center. Moffet Field. CA 94035. USA
[c]National Space Development Agency of Japan. Ibaraki. 305. Japan

Abstract

In this report, we describe the results of a rat pituitary cell culture experiment done on STS-65 in which the effect of cell feeding on the release of the six anterior pituitary hormones was studied. We found complex microgravity-related interactions between the frequency of cell feeding and the quantity and quality (i.e. biological activity) of some of the six hormones released in flight. Analyses of growth hormone (GH) released from cells into culture media on different mission days using gel filtration and ion exchange chromatography yielded qualitatively similar results between ground and flight samples. Lack of cell feeding resulted in extensive cell clumping in flight (but not ground) cultures. Vigorous fibroblast growth occurred in both ground and flight cultures fed 4 times. These results are interpreted within the context of autocrine and or paracrine feedback interactions. Finally, the payload specialist successfully prepared a fresh trypsin solution in microgravity. detached the cells from their surface and reinserted them back into the culture chamber. These cells reattached and continued to release hormone in microgravity. In summary, this experiment shows that pituitary cells are microgravity sensitive and that coupled operations routinely associated with laboratory cell culture can also be accomplished in low gravity.

Keywords: Rat pituitary cell culture; Microgravity

1. Introduction

If and how cells sense microgravity has been thoughtfully considered, modeled and debated for some time. Many different types of cells have been exposed to low gravity; some show significant and repeatable changes (Halstead et al., 1991; de Groot et al., 1990; de Groot et al., 1991; Limouse et al., 1991; Cogoli, 1993).

Our group has studied rat anterior pituitary gland cell structure and function during and after spaceflight (Hymer et al., 1992; Grindeland et al., 1987). We have focused on synthesis and release

* Corresponding author. Department of Biochemistry and Molecular Biology. The Pennsylvania State University, University Park. PA 16803, USA

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of growth hormone (GH) and prolactin (PRL) molecules because these two protein hormones are known to participate in the regulation of musculoskeletal, immune, vascular, metabolic and endocrine systems; systems which are often changed in low gravity. We recently reported that pituitary cells, in a passive cell culture system in low gravity, show differences in the quantity and quality (bioactivity) of GH and PRL released from primary rat pituitary cells in vitro (Hymer et al., 1996a; Hymer et al.. 1996b). Interestingly. some of these changes were similar to those found in pituitary cells prepared from spaceflown rats after 7–14 days in microgravity.

The unique design of the cell culture hardware available for this experiment permitted us to study, for the first time, possible effects of cell feeding on hormone release from each of the six major hormone-containing cell types. It also provided the astronaut the opportunity to prepare fresh solutions from preweighed powders and use them to trypsinize the anchorage dependent pituitary cells from their surface.

This experiment not only demonstrated that coupled technologies routinely associated with cell culture procedures on earth could be accomplished in the unique low gravity environment, but also showed that important microgravity changes in the quantity and quality of certain hormones released from this mixed cell culture occurred. In some cases, these changes were positively correlated with the frequency of cell feeding during 14 days in space.

2. Materials and methods

2.1. Animals and tissue processing before flight

Care and use of animals for this experiment that was done on the Space Shuttle in 1994 (STS-65) was approved by IACUC committees at Penn State and NASA Ames Research Center and conformed to NIH guidelines. Three days before launch, 150 specific pathogen-free Sprague Dawley male rats (200–220 gms, Harlan Sprague Dawley, Frederick, MD) were killed by decapitation and their anterior pituitary glands dissociated
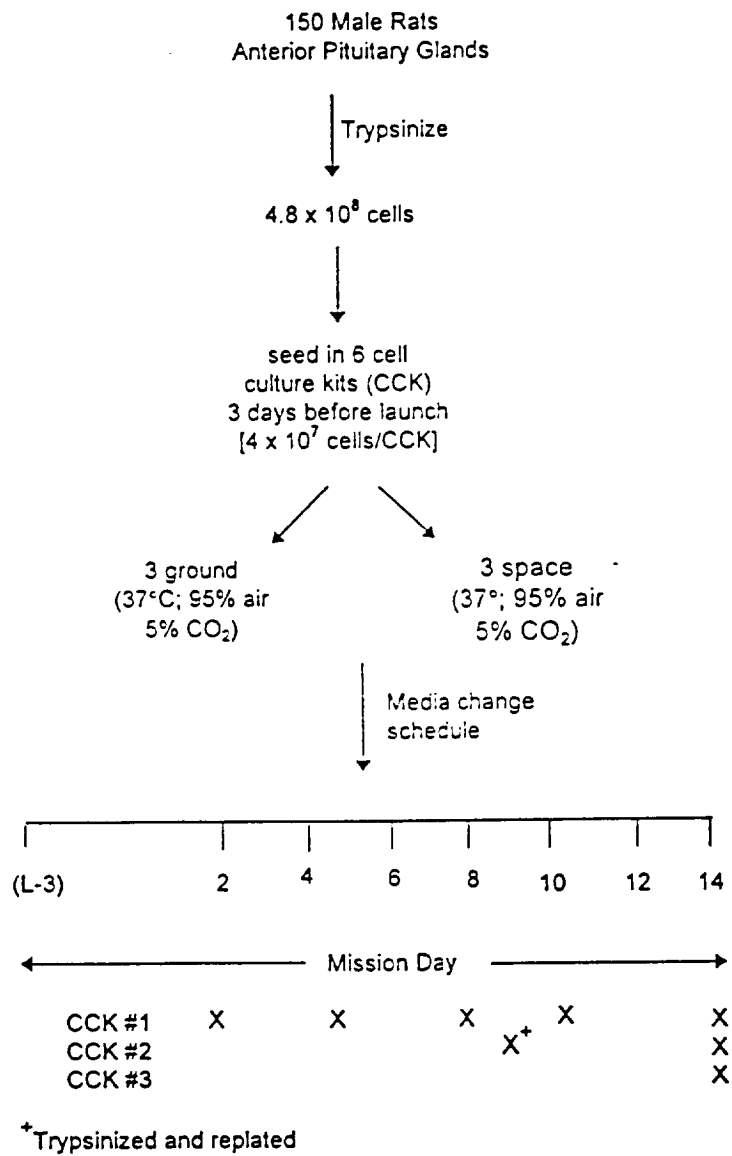
into a single cell suspension that routinely yields $2–2.5 \times 10^6$ cells from each gland (Hymer and Hatfield, 1983). For this experiment, total cells recovered were 4.8×10^8 with a viability > 95%. As shown in Fig. 1, 4×10^7 cells were seeded into each of six cell culture kits (CCK) specifically designed for cell culture technology in microgravity. Three CCK's were maintained in an incubator designed for spaceflight operations on Shuttle middeck while the other three were kept under similar conditions in the laboratory at Kennedy Space Center. Constant real time monitoring of operational parameters of this incubator in microgravity indicated that the temperature variance between the ground and flight units was ± 0.1°C; relative humidity ranged between 40–60% in both units throughout this 17-day experiment. The culture medium (15 ml CCK) was identical to that used in a previous rat pituitary microgravity cell culture experiment (Hymer et al., 1996a): viz. modified Eagle's minimum essential medium (αMEM) containing 5% calf serum. 0.2% NaHCO₃. 25 mM Hepes buffer (pH 7.4) and antibiotics.

Culture media in CCK #1 and #2 were changed according to the schedule shown in Fig. 1: cells in CCK #3 were left undisturbed over the entire course of the 17-day experiment.

2.2. Cell culture hardware.

The unique design of the CCK and its associated hardware permitted (a) microscopic observation of the primary rat pituitary cells attached to the pronectin-treated polycarbonate surface; (b) removal of spent media and their storage at −20°C; (c) addition of fresh culture media; and (d) on board preparation of two fresh solutions from preweighed powders stored in syringes (10 mg Difco 1:250 crude trypsin and 100 µg DNase (Type I, Sigma)).

Results of numerous preflight trials using pituitary cells in CCK's indicated that pretreatment with pronectin (10 µg/ml, Sigma) significantly helped promote cell attachment and also enhanced cell recovery after trypsin (data not shown). The appearance of a CCK and its operational configuration is given in Fig. 2. Basically,

150 Male Rats
Anterior Pituitary Glands

| Trypsinize

4.8 x 10^8 cells

seed in 6 cell
culture kits (CCK)
3 days before launch
[4 x 10^7 cells/CCK]

3 ground   3 space
(37°C; 95% air   (37°; 95% air
5% CO_2)   5% CO_2)

Media change
schedule

(L-3)   2   4   6   8   10   12   14

←———— Mission Day ————→

CCK #1   X   X   X   X$^+$ X   X
CCK #2   X$^+$ X
CCK #3   X

$^+$Trypsinized and replated

Postflight Analysis:

HPLC	Frozen Media	Cells
• Gel filtration • ion exchange	• hormone immunoassay • (GH, PRL, LH, FSH, TSH, ACTH) • hormone bioassays • (GH, PRL)	• flow cytometry • image analysis • immunocytochemistry

Fig. 1. Experimental design. See Materials and methods for details.

this unit consists of (1) a polycarbonate sheet (0.5-mm thick) to which the anchorage-dependent pituitary cells attach and (2) two chambers bounded by a 3.0-μ Nucleopore membrane which permits withdrawal and addition of culture medium from the chamber distal to the attached cells. The trypsin solution was prepared by passing 10 ml of a 5 mM Hepes buffered solution (containing 30 mM glycine, 0.2 mM potassium acetate, 0.5 mM MgCl$_2$·6H$_2$O, 0.03 mM CaCl$_2$, 220 mM glycerol, 44 mM sucrose and 0.2 mM ZnCl$_2$ and 0.2° EDTA), pH 7.26 into the syringe containing powdered trypsin (coupled luer fittings) to effect solution. After the removal of culture medium, this solution was then added to the attached cells in CCK = 2 (10 min) to promote detachment (monitored microscopically) followed by addition of 5 ml of a soybean trypsin inhibitor solution (1 mg ml) that had been prepared in Hepes buffer (above) before launch. The cells were then withdrawn from the CCK into the syringe containing powdered DNAase; this solu-
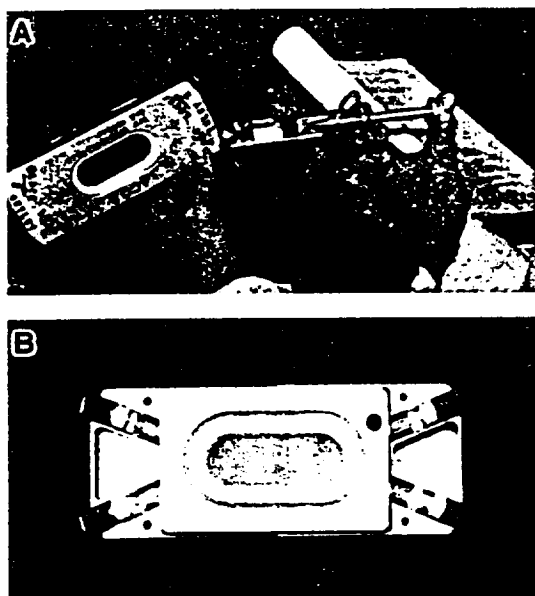

Fig. 2. Cell culture kit (CCK) used in this experiment. Syringes with luer fittings (A) enable media withdrawal as well as addition of new media; the actual culture chamber with its metal casing removed is also shown (B). See Materials and methods for additional details. (Photographs copied with approval from NASDA; taken from IML-2 brochure).

tion effectively breaks down nucleoprotein that may have been released from dead cells and routinely results in a smooth suspension of single cells (Hymer and Hatfield, 1983). After withdrawal of the trypsin-trypsin inhibitor solution into a storage syringe, the cells were reinjected into CCK #2 and 15 ml of fresh culture medium added for continued culture (Fig. 1).

2.3. Postflight analysis

Within 3 h of Shuttle landing, cells in each of the six CCK's were (a) photographed; (b) media withdrawn; (c) cells removed from CCK #1 and #3 by trypsinization and (d) cells in CCK #2 osmotically lysed by addition of dH$_2$O containing 0.2 mM ZnCl$_2$. The viabilities of cells removed from CCK #1 and #3 were > 80%. A portion (1 × 10⁶) of these cells were used for morphological analysis (see below); the remainder subjected to separation by free flow electrophoresis. Results from this later effort, as well as results of the electrophoretic separation trials of lysed cells, is published in the companion paper.

2.4. Morphology

Cells in the initial suspension, as well as those recovered at the end of the 17 day-culture period, were either (a) fixed and processed for immunocytochemical analysis of growth hormone (GH) and prolactin (PRL) cells or (b) multiparameter flow cytometric analyses exactly according to our established procedures that are described in several previous publications (Hatfield and Hymer, 1985; Perez et al., 1993). Parameters evaluated by flow cytometry (on 10 000 cells/sample) were (1) GH cell distributions; (2) marker indices, i.e. the ratio of the voltages of stained to unstained cells — an index of "brightness" of GH-specific fluorescence staining; (3) forward angle light scatter, FALS, (an index of cell size) and (4) perpendicular light scatter, PLS, (an indicator of content of cytoplasmic hormone-containing secretory granules). Procedures for the immunocytochemical analyses of GH stained cells attached to poly-l-lysine coated cover slips were exactly as described previously (Hatfield and Hymer, 1985); viz. fixation in Zam-

boni's fluid followed by membrane permeabilization with 0.4% Triton-x-100; incubation in GH-specific polyclonal antiserum followed by secondary amplification with horse radish peroxidase antiserum. These cell preparations were used for digital analysis of the cytoplasmic area occupied by GH using procedures and equipment identical to those used previously (Hymer et al., 1992).

2.5. HPLC

Two different types of chromatography were done to analyze the molecular nature of the immunoreactive GH (iGH) contained in the culture media taken from flight and ground CCK # 1. These were gel filtration chromatography to estimate apparent molecular weights of released iGH (Hymer et al., 1996a) and anion exchange chromatography to evaluate overall charge character of the released iGH. Samples from CCK # 2 and # 3 were not analyzed. In all cases, 1-ml aliquots of culture media were lyophilized, and reconstituted in either 500 μl of 0.1 M potassium phosphate buffer containing 0.05 M NaCl. pH 7.8 (gel filtration) or 250 μl of 200 mM TrisHcl. pH 7.8 for anion exchange chromatography.

For gel filtration, each sample was applied to a column of Protein-Pak SW (7.8 mm × 300 mm, Waters, Milford, MA) equilibrated with the same buffer. The column flow rate was 0.3 ml min. The column was calibrated with blue dextran (2 000 000 MW), beta-amylase (200 000 MW), bovine serum albumin (66 000 MW), carbonic anhydrase (29 000 MW) and ribonuclease (13 683 MW). The flow rate of the ion exchange column was 0.6 ml min and used a 0.6 M NaCl gradient for elution.

2.6. Hormone assays. Growth Hormone (GH)

Concentrations of immunoreactive GH (iGH) released from cells into culture media were determined by enzyme immunoassay (Farrington and Hymer, 1987). The polyclonal antiserum to GH has a cross reactivity of < 0.3% to prolactin at the final dilution (1:80 000) used in the assay; each sample was analyzed in duplicate at two dilutions and results are expressed relative to a rat GH standard preparation (B-11) kindly provided by the National Institute of Diabetes, Digestive, and

Kidney Disease and the National Hormone and Pituitary Program (University of Maryland School of Medicine).

Concentrations of biologically-active GH (bGH) in culture media and extracts were determined exactly according to the tibial line bioassay procedure of Greenspan et al. (Greenspan et al., 1949). Approximately 200 hypophysectomized female rats, 26 days old at surgery, were used to assay samples using a four-point assay procedure (i.e. 4 rats dose 2 doses). The assay endpoint measures increases in tibial epiphyseal plate widths after four daily injections of hormone; it has a sensitivity of 1 μg and is specific for GH. Responses were compared to a bovine GH standard (1.5 U mg) calibrated against a USP standard; they are expressed in terms of an in-house preparation of rat GH (3.0 IU mg).

2.7. Prolactin (PRL)

PRL enzyme immunoassay was done exactly as described previously (Signorella and Hymer, 1984) using polyclonal antiserum (cross reactivity to GH < 0.3% at a dilution of 1:40 000). Each sample was analyzed in duplicate at two dilutions and the results are expressed relative to a rat PRL standard (B-7) kindly provided by the National Institute of Diabetes, Digestive and Kidney Disease and the National Hormone and Pituitary Program (University of Maryland School of Medicine).

PRL bioassay was done by the Nb-2 lymphoma cell assay originally described by Tanaka et al. (Tanaka et al., 1980) and used routinely in our laboratory (Hymer et al., 1996b). This cell culture bioassay is specific for PRL and has a sensitivity of 0.2 ng ml. It is based on the ability of PRL to cause division in a line of T-lymphocytes prepared from lymphomas of estrogenized rats.

2.8. Follicle stimulating hormone (FSH), luteinizing hormone (LH), thyroid stimulating hormone (TSH) and adrenocorticotropic hormone (ACTH) assays

Radioimmunoassay kits for each of these pituitary hormones were kindly supplied by the Na-

tional Institutes of Diabetes, Digestive, and Kidney Disease and the National Hormone and Pituitary Program (University of Maryland School of Medicine). All hormones were iodinated using IODO-GEN (Pierce Chemical, Rockville, IL); 2.5 μg of purified hormone and 250 μCi of Na^{125}I. Assay protocols were exactly as described in NIDDK technical notes. The following antisera and reference preparations were used: (1) rTSH; iodination preparation rTSH-I-9 (AFP-1308C), reference preparation rTSH-RP-2 (AFP-5153B), antisera anti-rTSH (C21381); (2) rLH: iodination preparation rLH-I-7 (AFP-9404B), reference preparation rLH-RP-3 (AFP-7187B), antisera anti rLH-S-10; (3) rFSH: iodination preparation rFSH-I-8 (AFP-11454B), reference preparation rRP-2 (AFP-4621B), antisera anti rFSH-S-11 (AFP-C0972S8); (4) hACTH used in heterologous assay (rat reagent not available); iodination and reference preparation (AFP-632S031), antisera (AFP-2938C).

3. Results

3.1. In vitro hormone release in microgravity

The total quantity of immunoreactive GH, PRL, TSH, FSH, LH and ACTH released from the three different cell culture kits during 14 days in microgravity, relative to synchronous ground control cells, depended upon the frequency of media change and the particular hormone being measured (Fig. 3). Sometimes total hormone output from both ground and flight cultures was positively correlated with the frequency of media change, whereas in other cases feeding frequency had little effect. The total amounts of hormone released from either ground or flight cells varied over a wide range: i.e. ~10 micrograms (LH, FSH, TSH); 2–10 milligrams (PRL, GH); ~100 nanograms (ACTH). Noteworthy flight-associated differences in total hormone release were (1) a 4 × increase in GH release from unfed flight cells (Fig. 3A); (2) slight to moderate reductions in LH release from fed cultures (Fig. 3E); (3) small increases in GH and PRL release from cells fed four times (Fig. 3A and B); and (4) two-fold increases

in total ACTH released from unfed cells or cells fed 4 × (Fig. 3F).

3.1.1. CCK #1

The kinetics of hormone release from cells fed 4 times sometimes revealed very different patterns in microgravity that were often dependent on feeding frequency and hormone type. For example. feeding cells 4 times during spaceflight resulted in approximately linear increases in rates of release of PRL (Fig. 4D), FSH (Fig. 5A) and ACTH (Fig. 5G); however, the rates of release of PRL. FSH and ACTH from corresponding ground control cultures was different in each case. On the other hand. the rates of release of GH (Fig. 4A) and TSH (Fig. 4G) were similar between ground and flight. LH release (Fig. 5D) was initially 2–3 × more from flight cells. but later in the mission these rates were the same as from ground cells.

3.1.2. CCK #2

These cells had their first medium change on day 9 followed by an immediate trypsinization procedure which resulted in cell detachment from the polycarbonate surface (based on microscopic observation in both ground and flight chambers). These were reinserted back into the same chamber and the culture allowed to continue for an additional 5 days. In every case, the rate of release of each of the six hormones was greater from ground cells after trypsinization (Fig. 4B, E and H; Fig. 5B, E and H). However, in flight this release pattern was either similar (FSH. LH. TSH) or opposite (ACTH. GH, PRL).

3.1.3. CCK #3

These cells were left undisturbed over the entire experiment. The only changes in these flight cells were (a) a 4 × increase in GH release (Fig. 4C) and (b) a 2 × increase in ACTH release (Fig. 5I).

When these same media from CCK's #1, 2 and 3 were assayed for their content of biologically active GH and PRL, interesting flight-related differences were found, both in total amounts of bioactive hormone released and in the kinetics of that release. In terms of total bGH release, there was a ~50% reduction from fed
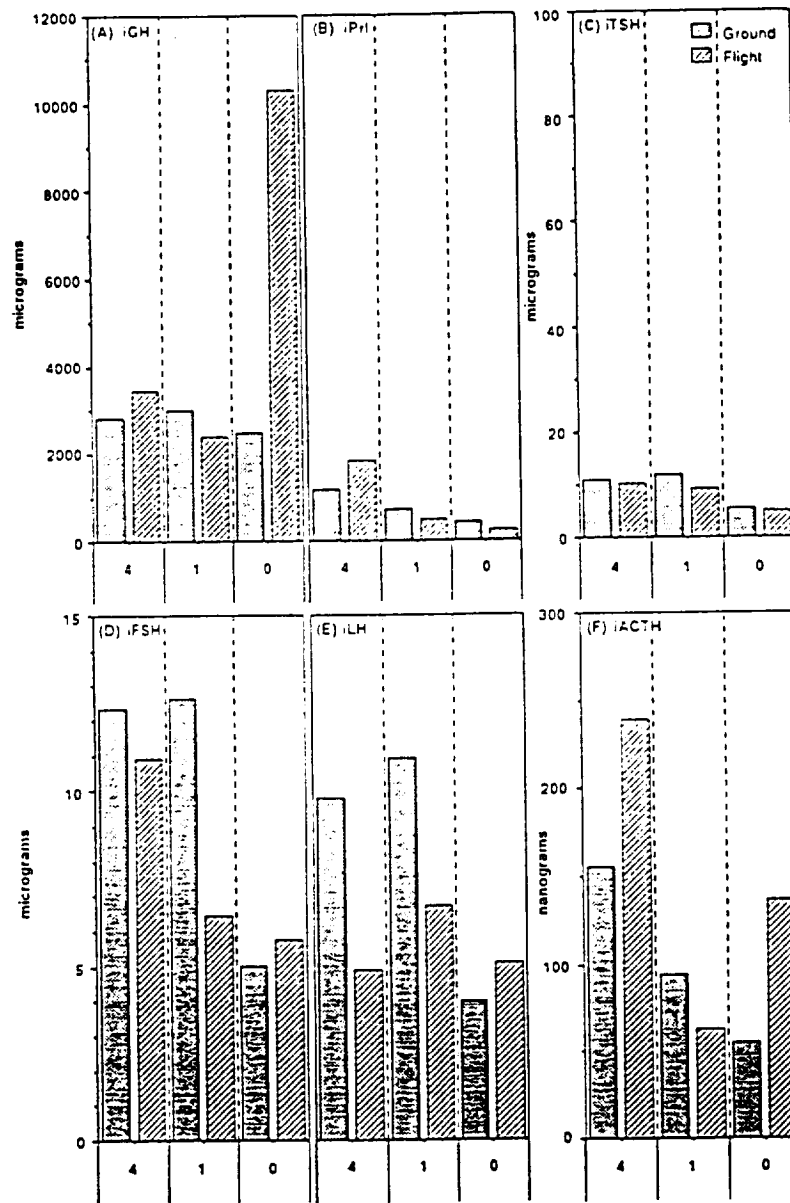
Fig. 3. Total release of immunoreactive (A) GH, (B), PRL, (C) TSH, (D) FSH, (E) LH and (F) ACTH from CCK # 1, # 2 and # 3 in which culture media were changed 4, 1 and 0 times, respectively, during 14 days in microgravity. Note large difference in total quantities of hormones secreted (micrograms-nanograms).

flight cells and a doubling of hormone from the unfed flight cells (Fig. 6A). In terms of total bPRL released, the situation was approximately reversed. Thus, there was a 2.5 × increase in total bPRL release from cells fed 4 times in microgravity while unfed PRL cells reduced bPRL output

by one-half (Fig. 6B). The kinetic data relating to release of bGH and bPRL indicated (a) a large burst in initial bGH from ground (but not flight) cells in CCK # 1 (Fig. 7A); (b) no correlation between amounts of iGH and bGH measured in CCK # 3 flight media (cf. Fig. 4C vs. 7C); and

finally (c) a good correlation between iPRL and bPRL contents in CCK # 1 flight media (cf. Fig. 4D vs. 7D).

3.2. Analysis of released GH by HPLC

Each of the five samples obtained from CCK # 1, after fractionation by gel filtration chromatography, contained two peaks of iGH with very different apparent molecular weights (Fig. 8). One of these approximated to the known mass of GH, viz 22 kd. The other had an apparent molecular weight $> 2 \times 10^5$. Neither coincided with the OD 280 profile which represents the major protein classes in the serum containing medium (Fig. 8, top). Generally speaking, the iGH elution profiles between the ground and flight samples
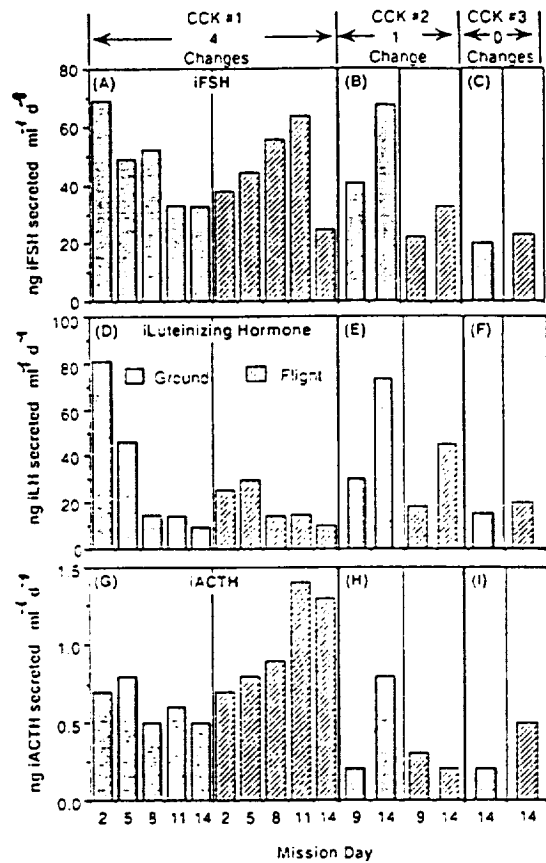

Fig. 5. Kinetics of release of iFSH, iLH and iACTH from CCK's 1–3 on earth and in microgravity.

were similar, although flight samples tended to have more low molecular weight hormone. Finally, there was a progressive loss of high molecular weight iGH from both ground and flight samples as the time of culture increased. Total recoveries of iGH from both ground and flight samples after this step averaged 46 ± 3% (n = 10). Of the recovered iGH, a majority was in the lower molecular weight region (range 56–81%).

After fractionation by anion exchange chromatography, each of the ten samples from CCK # 1 contained a single peak of iGH which eluted from the column before the salt gradient began. A majority of the OD 280 material also eluted in this same region (Fig. 9, top). While the amount of iGH from the flight samples remained relatively constant throughout the culture, there was

Fig. 4. Kinetics of release of iGH, iPRL and iTSH from CCK's 1–3 on earth and in microgravity.

a progressive loss of iGH from the ground sam-. ples. The reason for this pattern is unknown. Recoveries of iGH from both ground and flight samples (i.e. fractions 2, 3 and 4) after these steps averaged 88 ± 10% (*n* = 10) with no differences between ground and flight samples. Also shown in Fig. 9 (right column) are the biological activities of GH in fractions 2 and 3, assessed by the tibial line assay. Sometimes the patterns of bGH paralleled those of iGH (e.g. day 2, 5 and 14); other samples did not (e.g. day 11). It is especially interesting that the bGH concentrations were usually 2–7 × greater than those measured by immunoassay. Recoveries of bGH from both ground and flight samples averaged 373 ± 80% (*n* = 10) more than what was estimated to be present in the original sample of culture medium. This high recovery after ion exchange chromatography may result from removal of an inhibitor of bGH activity contained in the unfractionated sample.

The different profiles of iGH obtained after gel filtration and ion exchange chromatography
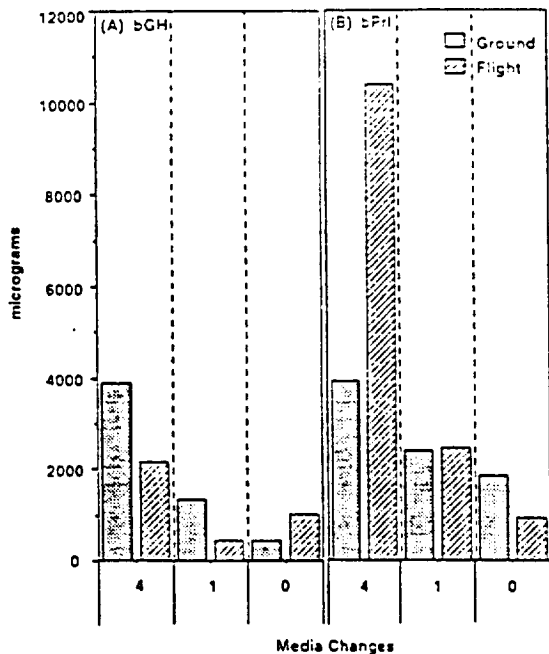
Fig. 7. Kinetics of release of bGH panels (A–C) and bPRL (panels D–F) from the 6 CCK's. Note scale difference.

prompted us to do a single rechromatography trial in which iGH molecules in fractions 2 and 3 were subsequently fractionated on the sizing column. Because supplies were limited, this trial was done only on the mission day 8 sample. The results show that what iGH was recovered (31% ground; 11% flight) had an apparent molecular weight in the range of 22 kd; none of the high molecular weight material in the original sample (Fig. 8) was detected in this region after rechromatography (Fig. 10).

3.3. Cell morphology

Phase contrast microscopy of cells in the flight CCK's obtained within 3 h after Shuttle landing showed that live (phase bright), attached cells were present in all ground and flight chambers.

Considerable care and time was taken to photograph the cell growth and distribution patterns in each CCK from random areas at several mag nifications. The photographs shown in Fig. 11 are taken at two different magnifications; they docu-

Fig. 6. Total release of bioactive GH (A) and bioactive PRL (B) from cells in CCK #1– #3 in which media were changed, 4, 1 and 0 times, respectively, during 14 days in microgravity.
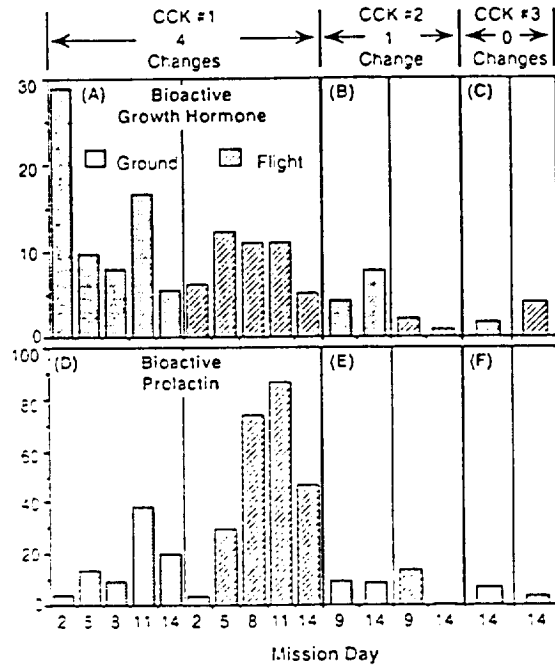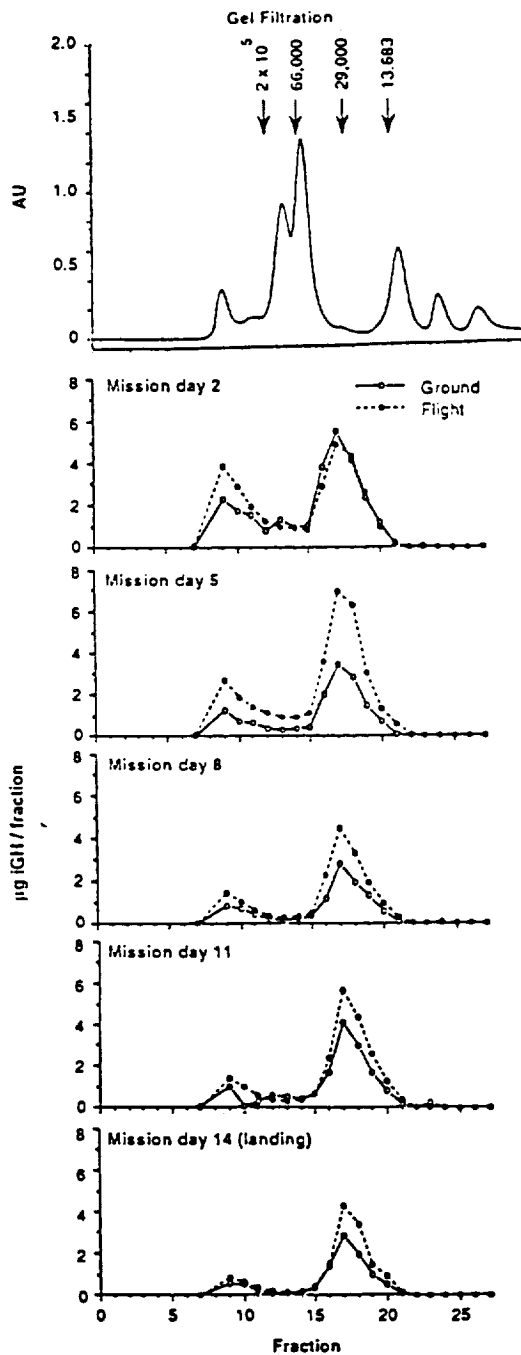
Fig. 8. Fractionation of culture media from spaceflown and ground control cells in CCK #1 by gel filtration HPLC. The optical density (OD 280) profile of serum proteins in the day-8 flight sample is shown in the top panel together with the elution position of standard molecular weight markers. OD profiles of all other samples were identical.

ment attachment, cell viability, and growth patterns that are representative of the entire culture. At low magnification, the dominant feature of cells in CCK #1 and #3 was their clumping in CCK #1, both ground and flight (Fig. 11A and B) and extensive clumping in flight CCK #3 (Fig. 11F) but not ground CCK #3 (Fig. 11E). Clumping of cells is easily seen because of scattered light which renders the cell clumps dark in the photomicrographs. Morphometry of these clumped areas from 3 different frames (entire photographic area) showed that these clumps occupied $10.9 \pm 1.0\%$ (CCK #1, ground): $12.6 \pm 2.6\%$ (CCK #1, flight): $6.7 \pm 0.5\%$ (CCK #3, ground); and $62.6 \pm 4.4\%$ (CCK #3, flight) of the total area. Cells in unclumped areas at higher magnification showed epithelial cell morphology that is typical for primary rat pituitary cultures except for one feature; i.e. total absence of fibroblast cell growth in unfed cultures from both flight and ground samples (cf. Fig. 11G and H vs. 11C and D). Comparison of Fig. 11C vs. 11D suggests that fibroblast growth is greater in microgravity, but other photographs (not shown) do not. The cell images in flight CCK #2, after Shuttle landing, indicated that these anchorage dependent pituitary cells, after trypsinization on day 9, reattached in microgravity sometime during the last 5 days of the mission (Fig. 12). These were more clumped than ground controls.

After Shuttle landing, our experimental design required cell removal from CCK #1 and #3 by trypsinization in order to do both cell image analyses as well as cell separation trials by continuous flow electrophoresis. The results of the electrophoresis trials are the subject of the companion report. In Fig. 13, we show the general features of trypsinized cells prepared from CCK's #1 and #3 (both ground and flight) after immunocytochemical staining with a GH-specific antiserum. At this level of discrimination, there were no obvious differences in GH cells (darker cells in Fig. 13) or non-GH cells between any of the treatment groups except for a few (< 10%) "giant" single cells (or remains thereof) in flight cells from CCK #3. Image analysis of immunocytochemically stained GH cells indicated that some
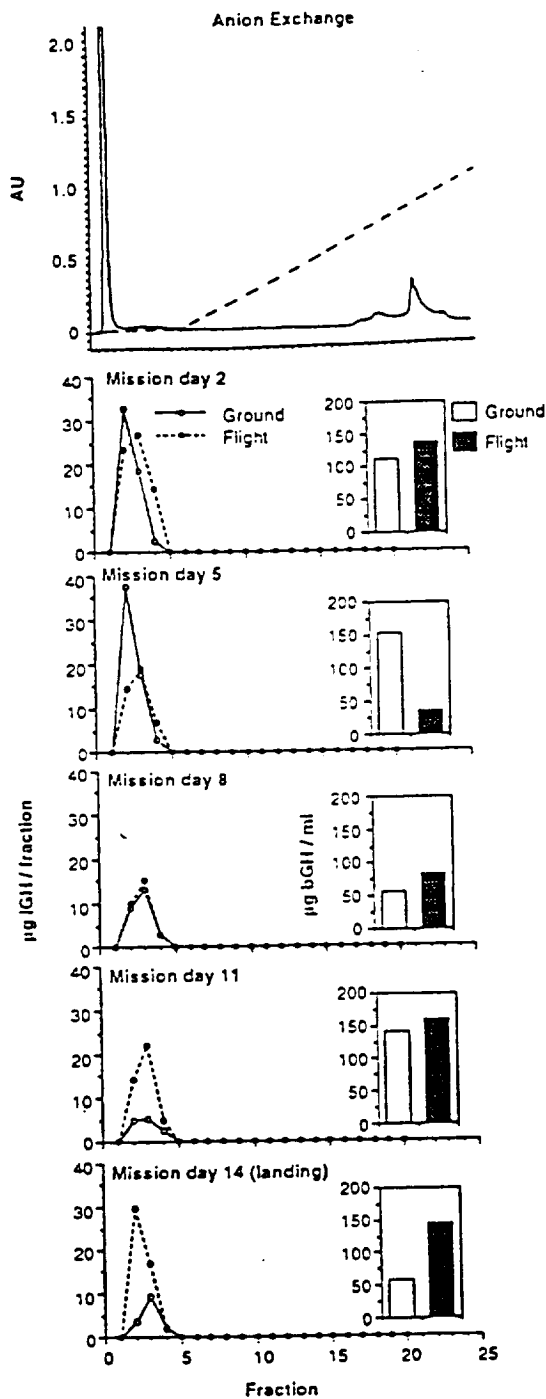
ground 28.0 ± 2.4%, flight 21.8 ± 2.1% (P < 0.05). The cytoplasmic area occupancy of GH in the initial cell suspension was 43.7%.

In Fig. 14, we compare GH staining and light scatter profiles of all cells (a) contained in the initial population at the beginning of the experiment (launch minus 3 days, L-3) to cells (b) after removal from CCK #1 and #3 17 days later. The top panel in Fig. 14 shows the distribution pattern of nucleated cells after staining with propidium iodide, a red fluorescent dye that intercalates with DNA bases and thus differentiates dividing from quiescent cells by fluorescence intensity. The single symmetrical peak of red fluorescence in the initial cell preparation at L-3 days is typical for post-mitotic primary rat pituitary cells (Fig. 14A). After the 17-day culture, there was a small population (22% ground, 11% flight) of cells from CCK #3 with > 2C amounts of DNA (Fig. 14B and C). These patterns probably represent dividing fibroblasts in these cultures. On the other hand, there was a considerable increase in the number of nucleated cells in CCK #1 which had > 2C amounts of DNA: 65% ground, 45% flight (Fig. 14D and E). Because many of these cells had a fibroblastic appearance after Shuttle landing (Fig. 11), the most plausible explanation is that this increased red fluorescence signal represents fibroblast growth induced by changing culture media.

The middle panels of Fig. 14F-J) show the pattern of GH cell staining identified by the gate in the figure. The well defined peak of green fluorescence in the initial cell preparation is typical (Hatfield and Hymer, 1985) and shows that 40% of the total population contained GH. It is interesting that the GH cell peak was better

Fig. 9. Fractionation of culture media from spaceflown and ground control cells in CCK #1 by ion exchange HPLC. The optical density (280 nm) profiles of all 10 samples were identical to the one shown at the top of the figure. A majority of OD 280 material eluted from the column before the 0.6 M NaCl gradient (dotted line) began. The left panels show the distribution profiles of recovered iGH; all of it was recovered from fractions 2, 3 and 4. GH bioassays of hormone contained in fractions 2 and 3 (right panel) indicated that more bGH than iGH was present in these two fractions. Although fractions #2 and #3 were assayed separately, only the combined total of bGH is graphed.
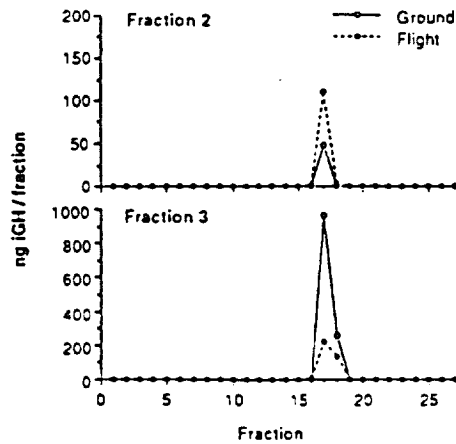
changes in the cytoplasmic areas occupied by GH had occurred: CCK #3, ground 28.3 ± 2.6%, flight 16.5 ± 2.1%, (P < 0.001); CCK #1,

Fig. 10. Two step fractionation of culture media from CCK #1. mission day 8. Fraction 2 and 3 material, after ion exchange HPLC (identical procedure to that done in Fig. 9) was followed by concentration and rechromatography by gel exclusion HPLC (identical procedure to that done in Fig. 8). The elution profile of the iGH shows that all of the recovered hormone had an apparent molecular weight of ~30 Kd. Note that none of the higher molecular weight iGH in another aliquot of this sample (Fr. #9. Fig. 8) was present after rechromatography.

defined in both flight samples: however, the marker index (an index of the "brightness" of GH fluorescence determined by the ratio of voltages of stained to unstained cells) was 50% greater in unfed flight cells from CCK #3 (8.6 vs. 5.7), but essentially no different in flight cells from CCK #1 (6.0 vs. 5.8).

The lower panels of Fig. 14 show the forward angle light scatter (FALS) profiles in the various samples; these are indicators of cell size. Initially, GH cells were larger than the other hormone-producing cell classes; after culture and trypsinization, each of the four cell samples tended to have smaller GH cells. However media changes tended

to result in increased mean FALS peak channel relative to unfed cells. This increase FALS signal from cells in CCK #1 was also found in the non-GH containing cell peak as well.

4. Discussion

A primary objective of this spaceflight experiment was to determine if the frequency of cell feeding affected either the quantity or quality (activity) of hormones released from cultured primary rat anterior pituitary cells during 14 days in microgravity. There is little doubt that this primary experimental objective was achieved. The versatility of the cell culture hardware, coupled with the ability to maintain temperature of the incubators on ground and space within 0.1°C, enabled us to obtain meaningful results. To our knowledge, this is the first report to describe the entire complement of profiles of the six predominant hormones released from cultured pituitary cells in microgravity.

In order to put the hormone release data into their proper context, it seems useful to make the following general statements concerning pituitary cell physiology in primary culture on earth. First, the intracellular concentration of GH in the pituitary is 80 × greater than the next most prevalent hormone (Lewis, 1992), a fact which explains the relatively high amounts of GH in the culture media (e.g. Fig. 3). Second, chemical factors which govern the release of anterior pituitary hormones from rat pituitary cells in earth culture are many and complex. For example, removal of the pituitary gland from chemical control of higher brain centers results in some loss of regulatory control of GH release (both positive and

Fig. 11. Phase contrast photomicrographs of cells in CCK #1 and #3 after the 17-day culture experiment. These micrographs were taken within 3 h of Shuttle landing. Images shown are representative of the morphology of the total cell population in CCK #1 (A–D) and CCK #3 (E–H) at two magnifications (original photographs taken at 4× magnification, panels A, B, E and F; or 20× magnification, panels C, D, G and H). The length of the micron bars is either 1000 μm or 100 μm for the low and higher magnification photographs, respectively. Differences in clumping between unfed cells in CCK #3 from ground (E) vs. flight (F) are obvious and they are representative of the entire field. The darkness of the image is caused by thickness of the cell clump and resulting light scatter. A higher magnification of the unfed cells from flight CCK #3 (panel F, arrow) is shown in Panel H. Growth of fibroblastic cells is obvious in flight CCK #1 (panel D); although not obvious, cells in ground CCK #1 (panel C); also had fibroblast growth. Code: CCK #1, ground (A and C), flight (B and D); CCK #3, ground (E and G), flight (F and H).
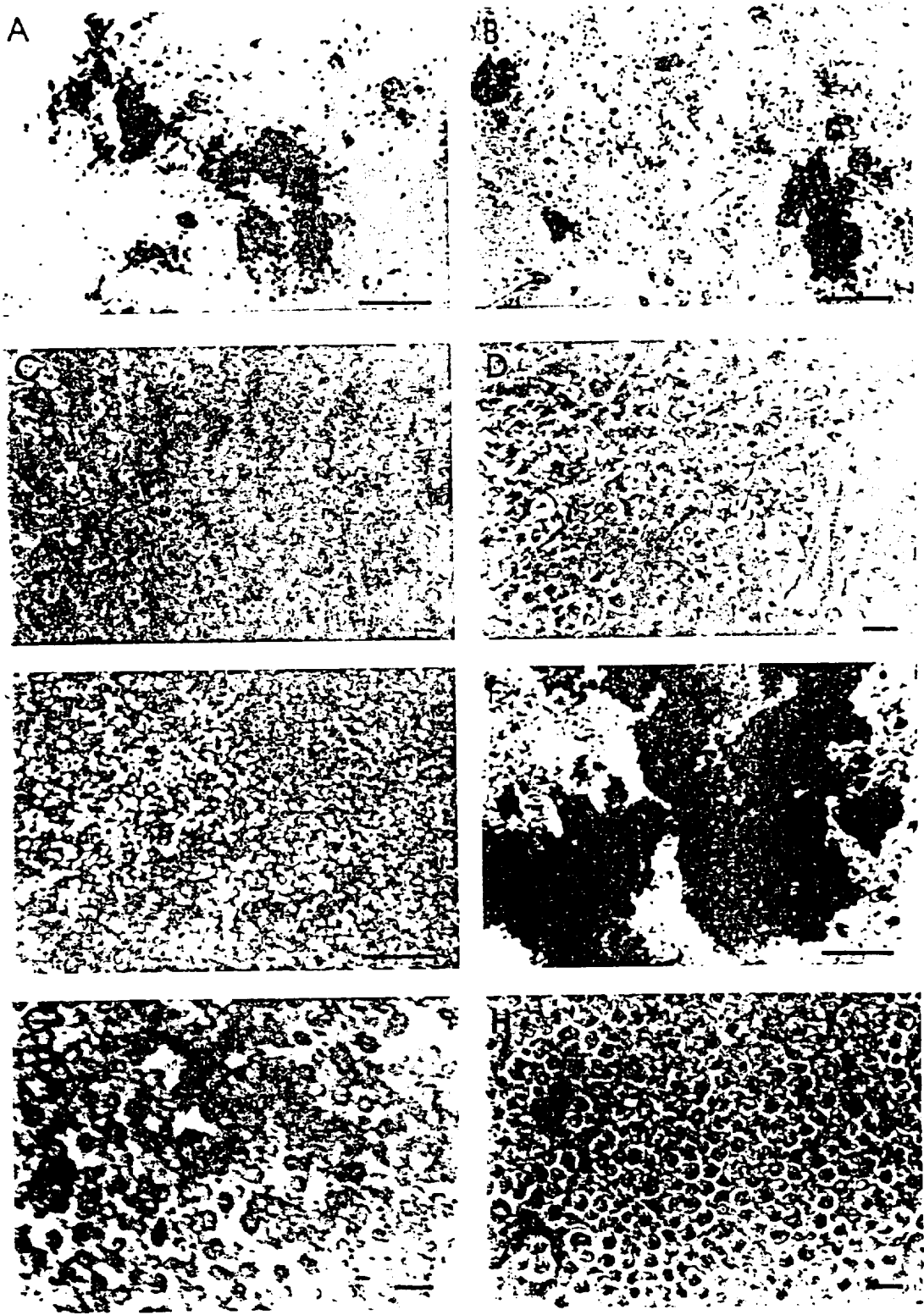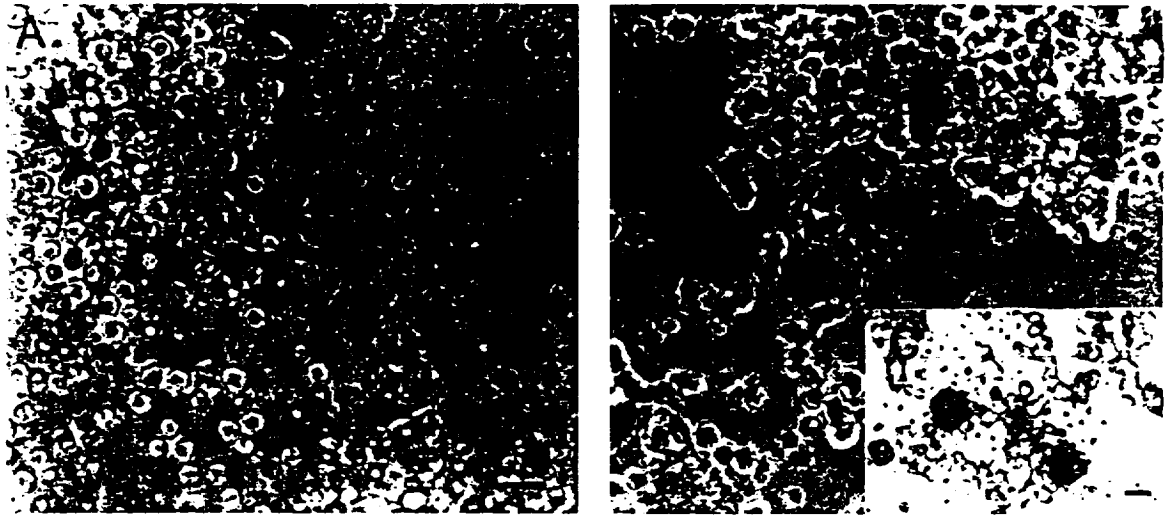
Fig. 12. Phase contrast photomicrographs of cells in CCK #2 after the 17 day culture experiment. As shown in Fig. 1, these cells had been trypsinized and detached from the polycarbonate surface of ground CCK #2 (panel A) or flight CCK #2 (panel B) on mission day 9 prior to reinsertion back into the CCK. Images shown are representative of their cell morphology seen after Shuttle landing. Cells from flight were more clumped. Insert (C) shows areas of aggregation and chains of epitheloid cells. All micron bars in this figure are 100 μm.

negative) as well as removal of a dominant inhibitory influence of dopamine from the brain on PRL release. The usual net result is "basal" release of preformed GH stores within GH cells and increased production (synthesis and release) of PRL in vitro (Wilfinger et al., 1979). Third, many hundreds of primary pituitary cell culture studies have been done in the last 25 years. In spite of the obvious differences in experimental variables such as medium formulation, cell density, cell surface attachment, autocrine paracrine interactions, time of culture, feeding frequency, potency of released hormone, general physiology and species of the pituitary donor, one can make certain generalizations concerning hormone release in vitro (Houben and Denef, 1990). For example, (a) in the absence of brain peptides, the synthesis and release of PRL continues unabated while the ex-

Table 1

Pituitary factors which are thought to control hormone release via autocrine or paracrine interactions[a]

Pituitary Factor	Autocrine (A) Paracrine (P)	Hormone System Affected
ATP	A P	LH
EGF	A	ACTH, TSH
Neurotrophic Factor	A P	ACTH(?)
VIP IGF-1	A P	PRL
GH,GH receptor binding protein	A P	GH, PRL, LH
Activin	A	FSH
HPLC Fractios from Gonadotropes	P	PRL, GH
Pituitary Adenylate Cyclase Activating Peptide	P	GH
Nitric Oxide	A P	LH, GH
Calcitonin	A P	PRL
Substance P	A P	LH
Arachidonic Acid	A P	LH

[a]Taken from literature searches covering last 3 year period. Representative Studies. See (Houben and Denef, 1990) for recent review.

Table 2

Correlation coefficients between total hormone released from CCK's 1-3 vs. number of media changes*

Hormone	Ground		Flight	
	Slope	r^2	Scope	r^2
iGH	+	0.17	–	0.36
bGH	+	1.00	+	0.70
iPRL	+	0.96	+	0.99
bPRL	+	1.00	+	0.99
LH	+	0.34	–	0.14
TSH	–	0.32	+	0.66
FSH	–	0.45	+	0.99
ACTH	–	0.98	+	0.61

*See Figs. 4 and 5 for data used to calculate correlation coefficients by regression analysis.

tent of GH synthesis is usually more modest; (b) hormone synthesis in the FSH, LH, TSH and ACTH cell systems does not occur to any appreciable extent unless the cells are properly stimulated in vitro; (c) paracrine interactions in the various hormone producing cell types take place as factors released from one type regulate hormone release from another (see Table 1); (d) evidence for autocrine feedback, i.e. decreased hormone release from a cell type caused by its prior release (e.g. PRL cells); and finally (e) the biological activity of a released hormone may not always be accurately reflected by immunological assays. This latter point is especially important because there is a growing awareness that hormone variants contained in the gland (variants resulting from, for example, aggregation, proteolytic cleavage, glycosylation, deamidation, phosphorylation of hormone molecules; Sinha, 1992) are also released in vitro. Some variants may go undetected in different assays.

How does the general pattern (i.e. directionality) of hormone release in microgravity compare to the usual behavior of earthbound cultured pituitary cells just described? Regression analysis of total hormone output, as a function of media change frequency, was used to address this issue (Table 2). If one assumes that a correlation of at least 0.7 represents a strong tendency towards an

effect, one can conclude that the overall patterns of release of some of the six hormones studied are not different in microgravity. Stated another way, the more frequently media are changed, the more bGH, iPRL and bPRL is released from both ground and flight cells. However, no such correlation is apparent for iGH, LH, and TSH from either ground or flight cells. In the case of FSH, there was excellent correlation between hormone release and cell feeding in flight, but not ground. In the case of ACTH, the correlation was high

Table 3

Parameters which suggest that microgravity-cell feeding interactive effects exist (top) relative to synchronous ground controls or do not (bottom)

| Parameter	Number of media changes	
	0	4
(1) Epithelial cell clumping	↑	Not different (ND)
(2) Cell size (non-GH cells)	ND	↑
(3) Cell size (GH cells)	↓	↑
(4) Total GH release pattern		
· iGH	↑ (314%)	↑ (22%)
· bGH	↑ (135%)	↓ (45%)
(5) Non-GH release patterns (total)		
· iPRL	↓ (45%)	↑ (57%)
· bPRL	↓ (50%)	↑ (165%)
· iLH	↑ (28%)	↓ (50%)
· iFSH	↑ (10%)	↓ (10%)
· ACTH	↑ (80%)	↑ (200%)
(1) Fibroblast growth	ND (none)	ND (extensive)
(2) GH cells		
· Cytoplasmic area occupancy	↓ (41%)	↓ (22%)
· Marker index	↑ (51%)	↑ (4%)
(3) Non-GH release pattern (total)		
· iTSH	ND	ND
(4) GH molecules		
· size	–	ND (↓ High MW)
· ion exchange		
iGH	–	Fluctuating
bGH	–	Fluctuating
· GH binding protein	–	?
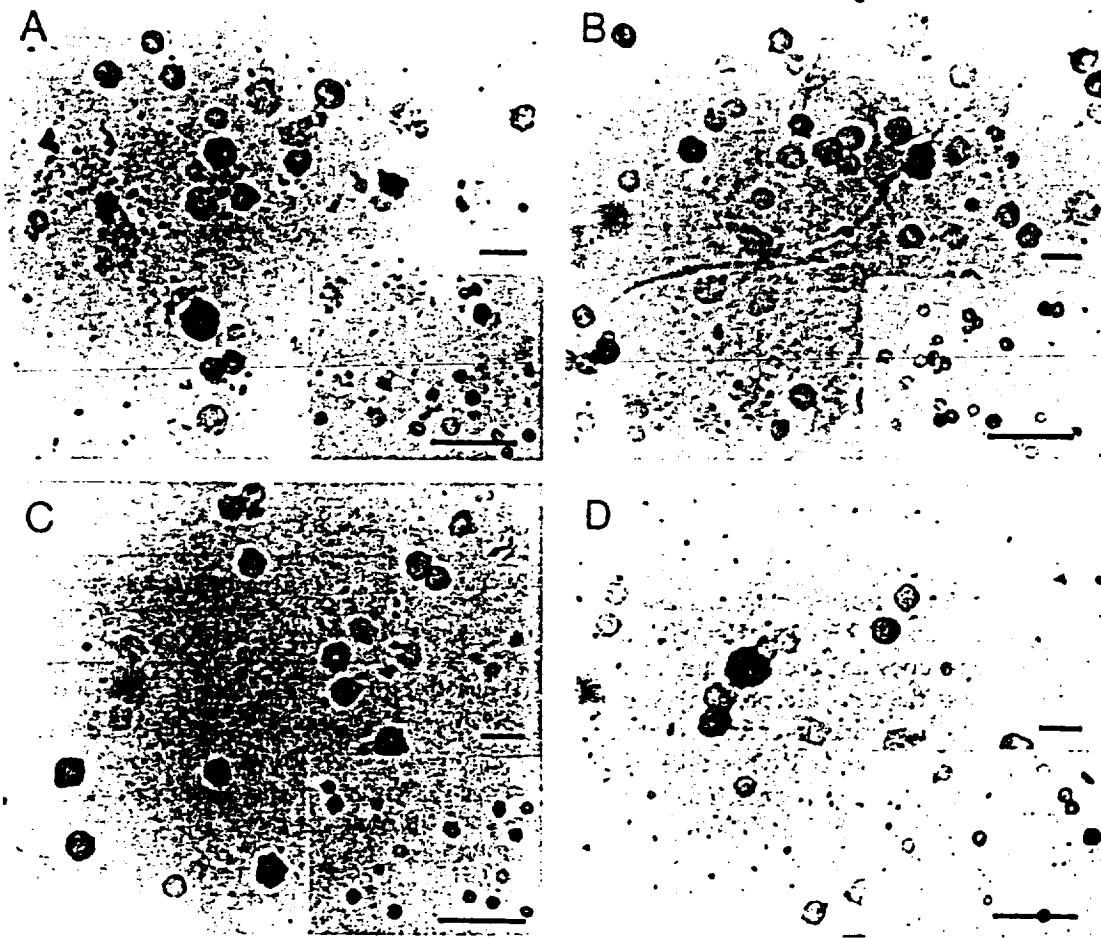
Fig. 13. Photomicrographs of cells recovered from ground and flight CCK's #1 and #3 after 17 days in culture. These cells were removed from the CCK's by trypsinization. allowed to attach to poly-l-lysine treated glass coverslips for 1 h prior to processing for immunocytochemical staining for intracellular GH (see Methods). Cells with dark cytoplasms are GH cells. Inserts are lower magnifications of different areas; all micron bars in the figure are 100 μm. Sample identification: (A) CCK = 1-ground; (B) CCK #1-flight; (C) CCK #3-ground; (D) CCK = 3-flight. Note the fibroblastic appearance of some cells in panel B.

from ground. but not flight cells. As shown in Table 2. the directions of change. reflected by slopes of the regression lines. were actually opposite in sign between ground and flight in two instances (iLH. iGH). These findings obviously reflect the complexity and specificity in behavior of hormone release from this heterogeneous cell system.

These generalizations should not be interpreted to indicate that the quantity or quality of the hormone is unaffected by feeding frequency in microgravity. Indeed. some microgravity-specific changes are clearly evident in flight cells as

reflected in (1) a large increase in iGH and ACTH release from unfed cells: (2) moderate reductions in LH and FSH release from fed cells: (3) modest increases in iGH and iPRL release from cells fed four times: and finally (4) a large increase in bPRL release from cells fed four times in spaceflight.

What might account for these microgravity-associated changes in hormone release? The most obvious operational difference between cells in CCK #1 vs. #3 is the frequency of medium replacement. Lack of feeding will result in a consistently quiescent environment that cells in flight
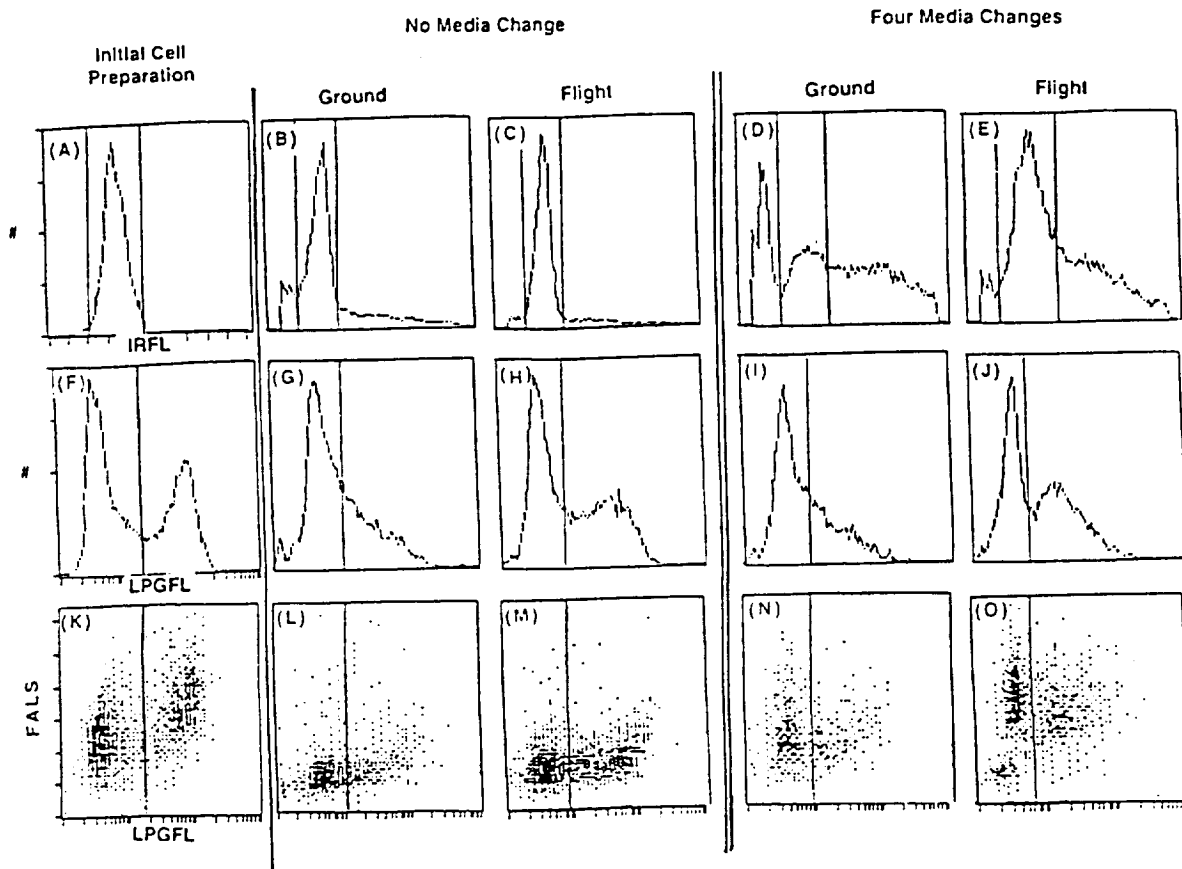
Fig. 14. Flow cytometry profiles of pituitary cells prior to seeding into the 4 CCK's at L-3 days (panels A, F and K) or after recovery from CCK ≠ 3 (panels B. C. G. H. L and M) or CCK ≠ 1 (panels D. E. I. J. N and O) 17 days later. The top row of panels show profiles red fluorescence after staining with propidium iodide. a DNA marker which indicates the concentration of DNA cell. Increased fluorescence in cells from CCK ≠ 1 may reflect fibroblast growth and division in this sample. The middle panels show GH-stained cells (green fluorescence). The profile in the initial cell sample (panel F) is typical and differentiates stained from unstained cells. Note that the GH staining profiles were better defined in the flight samples (panels H and J). The lower panels show the forward angle light scatter (FALS) patterns of unstained (arrow, panel K) and GH stained cells before and after flight.

CCK #3 must have experienced. The excellent theoretical analysis by Albrecht-Buehler (Albrecht-Buehler, 1991) considers possible consequences of such a quiescent environment on mammalian cell function in microgravity. How might such an environment actually result in the differences found in our study? To address this problem, we searched for results that were either opposite in character (or very different in magnitude) between CCK #1 vs. #3, because these presumably would be of most interest in sorting out microgravity-feeding frequency interactions. Differences meeting these criteria are given in Table 3 (top); those which do not are shown in

Table 3 (bottom). Because evidence for the importance of autocrine/paracrine interactions in regulating hormone release from anterior pituitary cells continue to grow (Table 1), and because decreased microconvection in pituitary cell cultures in spaceflight could conceivably impact on these types of interactions, it seems reasonable to postulate that some of the changes we have found in this experiment can be explained in part by the models offered in Fig. 15 and 16.
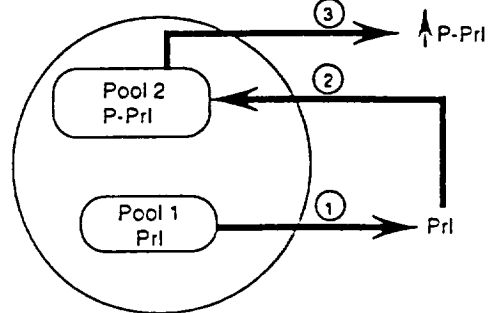
An example of hormone release from a cell class controlled by an autocrine feedback loop is the PRL cell. Walker's laboratory has shown that PRL released from a cell in culture can feedback
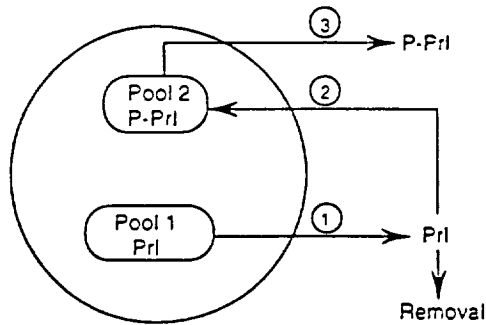
PROLACTIN CELL IN VITRO

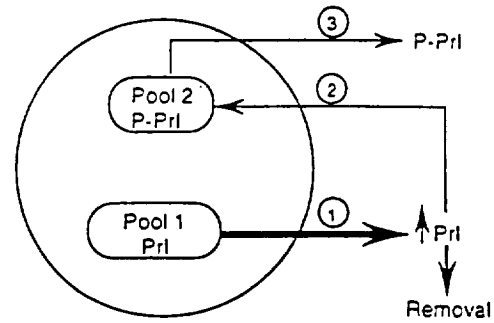Ground - no medium change   Flight - no medium change
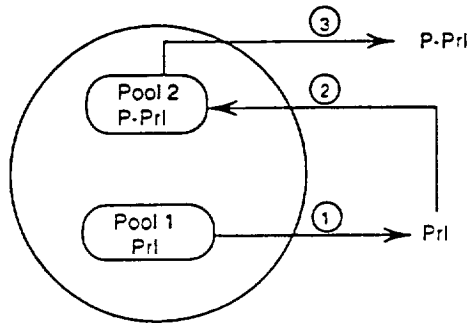
EVENTS IN MICROGRAVITY

(A)   Prl cell "senses" new environment; ↑ Prl buildup around cell

(B) $\dfrac{P\text{-}Prl}{Prl} > 1$

(C)   Result; ↓ Activity of Prl in Nb-2 cell bioassay

Ground - medium change   Flight - medium change

EVENTS IN MICROGRAVITY

(A)   Prl cell "senses" new environment

(B)   Non-phosphorylated Prl removed via medium change

(C)   ↓ Autocrine feedback

(D) $\dfrac{P\text{-}Prl}{Prl} < 1$ Result; ↑ Activity of Prl in Nb-2 cell bioassay

Fig. 15. An autocrine feedback model for bPRL release.

on itself via an autocrine loop; ultimately this modifies the molecular form of the hormone (in this case its degree of phosphorylation) via post-translational control mechanism(s) (Walker,
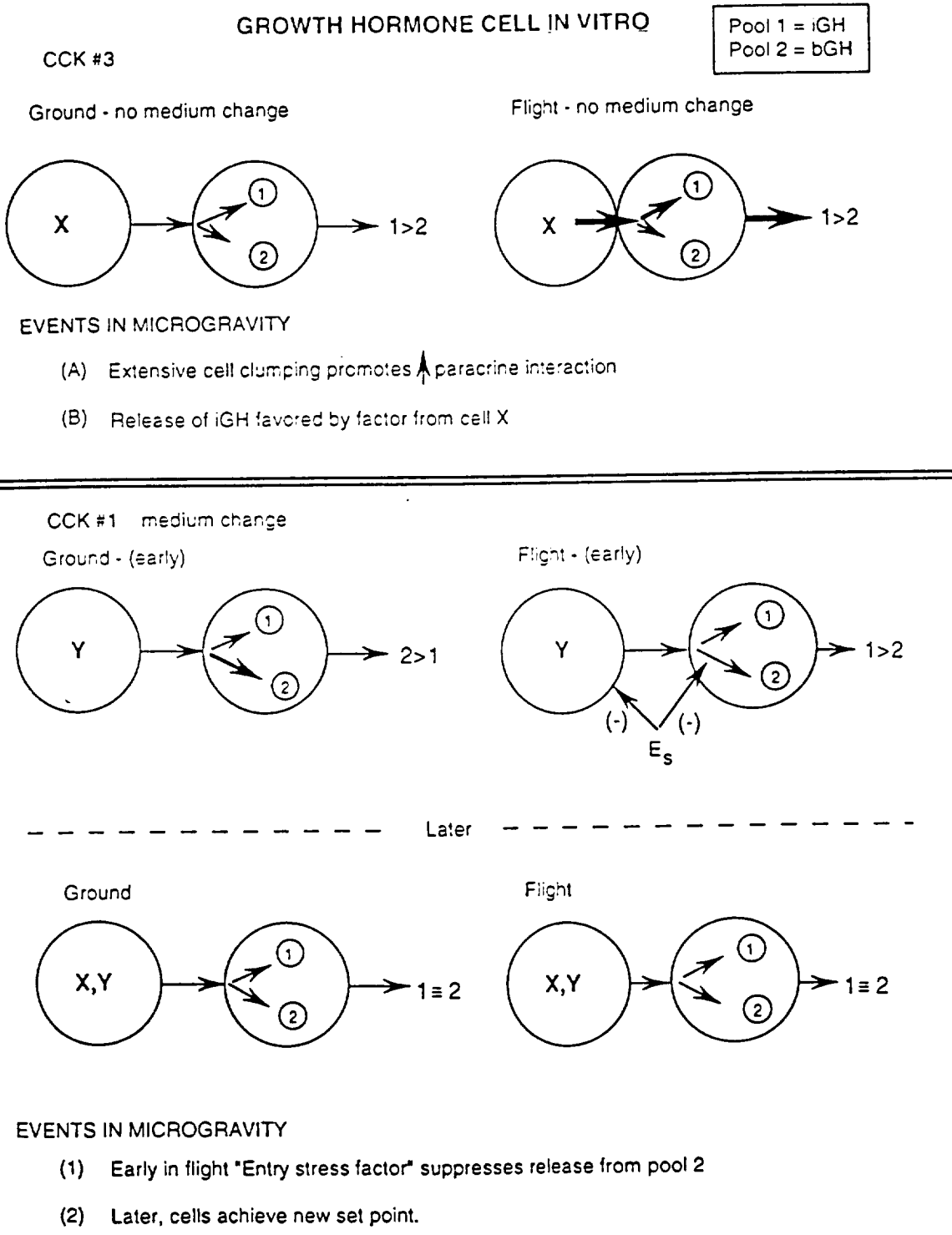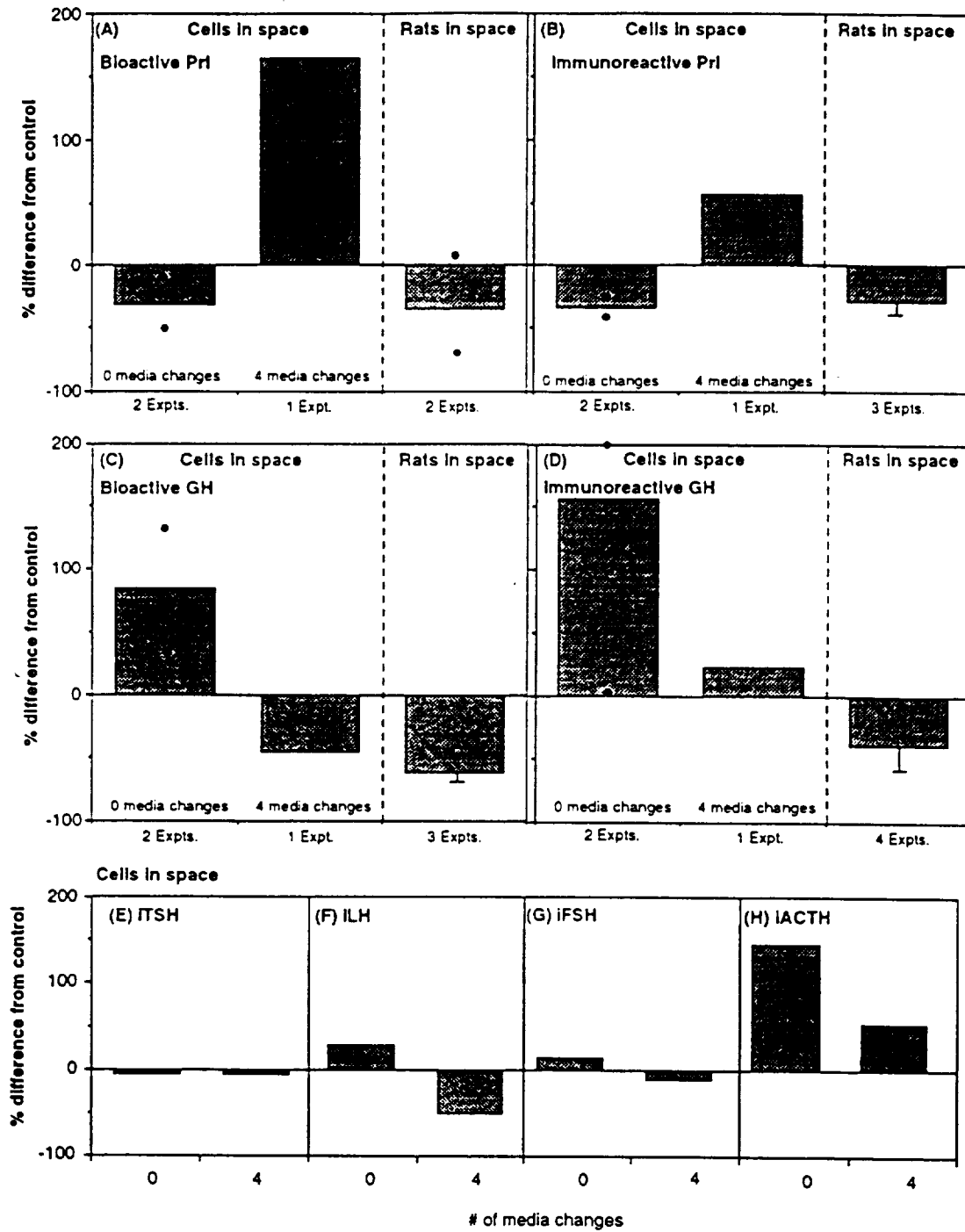
GROWTH HORMONE CELL IN VITRO

CCK #3

Pool 1 = iGH
Pool 2 = bGH

Ground - no medium change

Flight - no medium change

EVENTS IN MICROGRAVITY

(A) Extensive cell clumping promotes ↑ paracrine interaction

(B) Release of iGH favored by factor from cell X

CCK #1 medium change

Ground - (early)

Flight - (early)

(-) (-)

E_s

— — — — — — — — — — Later — — — — — — — — — — — — —

Ground

Flight

EVENTS IN MICROGRAVITY

(1) Early in flight "Entry stress factor" suppresses release from pool 2

(2) Later, cells achieve new set point.

Fig. 16. A paracrine feedback model for bGH release.

1994). We suggest that PRL cells in CCK #3 in microgravity have augmented autocrine feedback resulting directly from an increased accumulation of PRL around the cell. As suggested by the model (Fig. 15), the extracellular fluid environment might accumulate molecules having a ratio of phosphorylated PRL (p-PRL) PRL > 1. Because p-PRL is less active in the Nb-2 cell bioassay (and indeed can neutralize the activity of non phosphorylated PRL in this assay in a ratio of 1 phosphorylated variant to 10 native molecules (Wang and Walker. 1993). the final result would be decreased activity of bPRL in media from flight CCK #3. On the other hand. autocrine feedback in CCK #1 would be less as a consequence of medium changes: this might result in a p-PRL PRL ratio of <1. In turn. increased activity of PRL in flight CCK #1 media would result as suggested by the model and the actual data (Fig. 6).

How paracrine interactions might be affected by the microgravity environment was suggested by results of our GH cell studies on earth and in space. For example. we have shown that approximately one-half of all GH cells have densities > 1.070 g cm³ (Snyder et al.. 1977). Functional and biochemical differences in GH molecules released from these dense GH cells in vitro include (a) greater amounts of disulfide-linked oligomeric forms (Farrington and Hymer. 1990) and (b) greater biological activities in the hypophysectomized rat tibial line assay (Hymer et al.. 1981). A convincing demonstration that paracrine interactions can affect the quantity and bioactivity of GH released from cells in microgravity was obtained in our passive 8-day pituitary cell culture spaceflight experiment in 1992 (Hymer et al., 1996a). In that experiment. we showed that release of bGH from dense GH cells. in isolation, was reduced by ~50% relative to ground control

cells. However. in mixed cell cultures. output of bGH in microgravity was significantly increased. This finding established that some factor(s) released from cells in the less dense cell population stimulated the dense GH cells to release more bGH in microgravity than on earth. The identity of the paracrine stimulatory molecule(s) remains elusive: as suggested by the list in Table 1. it could be GH itself (an isoform?); a GH receptor binding protein made within the pituitary gland or another factor (other hormones?). How might the feeding frequency of GH cells in microgravity affect paracrine regulators and ultimately the quality of released GH? The massive cell clumping seen in flight CCK #3 (Fig. 11) could enhance paracrine interactions leading to increased release of bGH (Fig. 6). Although decreased convection in flight CCK #1 experienced during the 3-day intervals between media changes might be expected to minimize effectiveness of paracrine interactions (Fig. 16). the kinetic data show that the most important effect occurred on day 2: the other four samples from both ground and flight contained approximately the same amount of bGH. Other additional mechanisms must certainly be involved (e.g. "stresses" associated with launch) to account for changes we find in hormone release. As suggested by the model. several paracrine factors may be involved.

We have been fortunate to be able to do two pituitary cell culture experiments in space as well as three experiments using pituitary cells from spaceflown rats. Their focus has been exclusively on GH and PRL. It is interesting to compare the results from these in vitro vs. in vivo approaches, but given variability in experimental design (e.g. length of time in microgravity) the comparisons can of course be taken only so far. Nevertheless, comparisons of bPRL (Fig. 17A) and bGH (Fig. 17B) release are interesting. Clearly, four media

Fig. 17. Comparison of bPRL (A and B) and bGH (C and D) release from either mixed pituitary cell cultures in microgravity or from mixed pituitary cell cultures prepared postflight from spaceflown rats. The two experiments using passive (unfed) cell culture were in 1992 (STS-46, 8 days in microgravity) and in 1994 (STS-65; 14 days in microgravity, CCK #3, this report); the single experiment employing four media changes was also STS-65. The three experiments using spaceflown rats were SL-3 (7 days); COSMOS 1887 (13 days) and COSMOS 2044 (14 days). Release of bPRL from spaceflown rats was only done on the 2 COSMOS flights. Dots represent values of individual experiments; error bar = S.E.M. Similar comparisons between the release of immunoreactive TSH. LH, FSH and ACTH from fed and unfed cultures. relative to ground controls. are shown in panels E-H. None of these latter hormones were measured in cells from our spaceflown rats (Hymer et al.. 1992; Grindeland et al.. 1987).

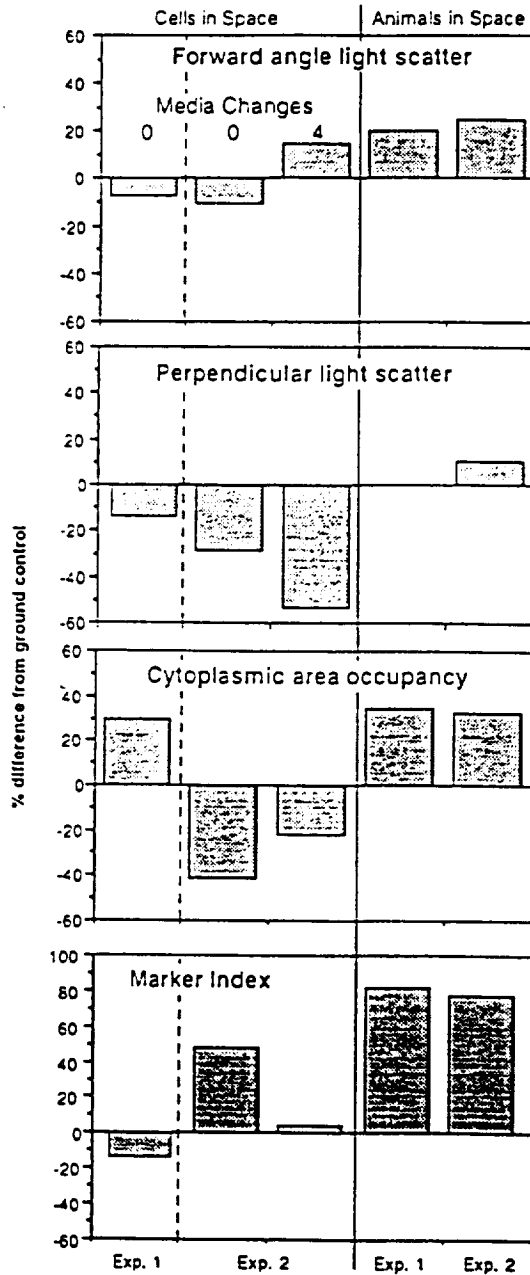changes in low gravity result in very different release profiles of bioactive PRL and GH. The

Fig. 18. Biophysical parameters of GH cells after cell culture in microgravity (left panel) or after their postflight preparation from spaceflown rats (right panel). Cell culture: Exp. # 1 was STS-46; # 2 was STS-65 (this report). Rat spaceflight Exp. # 1 was COSMOS 1887; Exp. # 2 was COSMOS 2044.

fact that four media changes in flight yielded bGH changes that were of a magnitude similar to that from cells of the intact animal would tend to argue for better "physiological fidelity" when culture media are changed. However, that same idea does not seem to apply for bPRL. Once again, specificity of microgravity effects in terms of different hormone release behavior becomes evident. Although less complete, the summary data in panels C-F of Fig. 17 also show different release patterns of TSH, LH, FSH and ACTH in microgravity as a function of cell feeding.

The "negative" information in Table 3 (bottom) is also important in the sense it shows that some of the cellular mechanisms at play in the ground cultures are also operational in flight. These include the finding (1) that pituitary cells were able to reattach to the surface of the CCK ≠ 2 after trypsinization; these reattached cells continue to release hormones, often at a comparable rate to the ground control cells; (2) that the mechanism(s) which promote fibroblast growth in fed cultures on earth also appear operable in microgravity; and (3) that the molecular characteristics of the released GH, at our current level of biochemical analyses, are not different between the two groups. We are not the first to show that anchorage dependent cells can reattach in microgravity (Morrison, 1994). Moreover, our previous data, while not as extensive as those collected in this experiment, also failed to find significant microgravity-related changes in apparent size of released GH molecules (Hymer et al., 1996a). If molecular changes do in fact occur, they will obviously require probes different from the ones used to date. However, we do consider the preliminary data regarding the behavior of released GH from the mission day-8 sample after rechromatography interesting and potentially important. We favor the interpretation of these data (Fig. 10) to indicate that a portion of secreted GH is bound to carrier. However, we cannot discount the possibility that this high molecular weight GH represents aggregated hormone. If this idea is valid it would mean that production of this molecular complex decreases as the culture period progresses. Future experiments directed toward this aspect of the problem may prove worthwhile. The nature of

bGH molecules which are released from the pituitary gland of the human and rat has been a topic of much interest in our laboratories for many years. The fact that recoveries of tibial growth promoting activity after the gel filtration step ranged 40–80% of that measured in the original culture fluid, but ranged 200–400% after ion exchange chromatography suggests that an inhibitory material may be co-released which obviously would result in an underestimation of the actual concentration of bGH in the unfractionated sample.

Data from this experiment are internally consistent with the Albrecht-Buehler's hypothesis that extracellular medium quiescence represents the primary gravisensing mechanism which will lead to altered hormone release (Albrecht-Buehler, 1991). What are likely secondary biophysical changes inside the cell that would be called into play after this primary gravisensing event occurred? The four cellular markers studied in GH cells thus far (Fig. 18) yield results that are interesting, but not definitive. Changes in cell size (FALS) might indirectly reflect the changes in tensional integrity of the cytoskeletal network. Furthermore, the different character of the GH fluorescence intensity measurements in some of the in vitro vs. in vivo situations may reflect differences in intracellular packaging such that epitopes on the GH molecules are exposed to a greater or lesser degree in spaceflown cells. Such changes would manifest themselves as differences in fluorescence intensity. The other measurements represented in Fig. 18, viz cytoplasmic area occupied by GH and perpendicular light scatter signals, bear on the distribution of the intracellular secretory granules (Hatfield and Hymer, 1985). It is not obvious how they correlate with the hormone release data.

Operations in biotechnology laboratories on earth are routinely coupled, one process with another. This spaceflight experiment successfully demonstrated that it is possible to change cell culture fluids in sterile fashion: freeze the spent medium; prepare a fresh trypsin solution in microgravity; trypsinize and reseed cells — all operations that will certainly be required on a routine

basis when the Space Station is operational.

The general conclusion that anterior pituitary gland function is affected in low gravity is inescapable: so too is the conclusion that a complete definition of the underlying cellular and molecular mechanisms will require extensive use of cell culture technology in microgravity. It would seem that any future pituitary cell culture experiment in microgravity should be tailored to meet the unique behavior of the primary hormone system being studied. When mechanisms are better understood, they should prove of use in studies aimed at defining countermeasures to physiological changes encountered during manned spaceflight. At the same time, they can serve the dual role of application of a unique environment to learn more about the function of this complex neuroendocrine system on earth.

In summary, this spaceflight experiment has provided significant new information concerning the issue of direct microgravity effects on pituitary cell structure and function in microgravity. From what we now know, there seems little question that some pituitary cell types directly "sense" the lack of gravity, regardless of whether they are present in their native condition (i.e. the rat) or in a cell culture system. In terms of secretory function, GH, PRL and ACTH cells appear to be the most sensitive of the six major hormone-producing cell types to the low gravity environment.

the manuscript. Finally, our sincere thanks to the crew of STS-65 for their help in making this experiment possible.

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