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**"ELECTROPHORETIC SEPARATION OF CELLS AND PARTICLES  
FROM RAT PITUITARY AND RAT SPLEEN"**

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# INTRODUCTION/SUMMARY

## 1.1 Description of Experiment

There are 3 parts to the IML-2 TX-101 experiment. Part 1 is a pituitary cell culture experiment. Part 2 is a pituitary cell separation experiment using the Japanese free flow electrophoresis unit (FFEU). Part 3 is a pituitary secretory granule separation experiment using the FFEU.

The objectives of this three part experiment are:

- to determine the kinetics of production of biologically active growth hormone (GH) and prolactin (PRL) in rat pituitary GH and PRL cells in microgravity ( $\mu g$ )
- to investigate three mechanisms by which a  $\mu g$ -induced lesion in hormone production may occur. Mechanism #1 tests the hypothesis that production of biologically active hormones in  $\mu g$  depends upon paracrine interactions between cell populations in the culture vessel. Mechanism #2 tests the hypothesis that secretory mechanism(s) in GH/PRL cells in  $\mu g$  are different than in cells in Earth's gravity (1g); this difference will be reflected in changes in electrophoretic mobilities of cells separated in  $\mu g$ . Mechanism #3 tests the hypothesis that  $\mu g$ -induced changes in hormone packaging within subcellular organelles will be reflected in differences in electrophoretic mobilities of these organelles electrophoresed in flight vs. ground.

- to determine the quality of separations of pituitary cells and organelles by continuous flow electrophoresis (CFE) in  $\mu\text{g}$  under conditions where buoyancy-induced convection is eliminated.

The experiments described herein will be accomplished in two pieces of flight hardware that have been designed and tested by NASDA. One of these is a cell culture kit (CCK) which not only permits cell photography in flight, but also allows change out of culture media and storage of the spent media for subsequent analysis at 1g. The second is a free flow electrophoresis unit that not only separates biological particles (cells/secretory granules), but also allows for continued cell culture and/or storage in  $\mu\text{g}$  prior to return to Earth. It is important to emphasize that the designs of these two pieces of flight hardware, for the first time, permit the experimenter to do reasonably complex sets of coupled methodologies in  $\mu\text{g}$  that are now done routinely in biotechnology labs on earth. A successful experiment will demonstrate that these new approaches can be useful to probe mechanisms by which  $\mu\text{g}$  affects cell function. As such, this biotechnology experiment serves as a precursor to similar experiments that will certainly be done on Space Station Freedom.

## 1.2 Scientific Knowledge to be Gained

In man, both GH and PRL are multifunctional hormones that are essential for life. While their existence has been known for 25-50 years, the biochemical details and functional significance of molecular events involved in their synthesis and release from the GH/PRL cell are still incompletely understood. Moreover, the body targets

for these two hormones are numerous. Our understanding of the ultimate consequences of binding of these hormones to different tissues is also incomplete.

It is obvious that the methodological approaches used to study mammalian pituitary cell function on earth have both advantages and disadvantages; the quality of an experimental result depends upon the limitations of the method(s) used and an awareness of those limitations (Appendix - Table 1). The PI's laboratory has used many of these different methodologies to develop this data base. Our current understanding of pituitary cell function establishes the complexity and heterogeneity of the pituitary GH and PRL systems. Heterogeneity exists in 1) the molecular forms (isoforms/variants) of GH and PRL; 2) the specialized subpopulations of GH/PRL cells that may process these isoforms differently; 3) the geographic location of these cell subpopulations within the pituitary gland and finally 4) in the biological potency of the secreted hormone variants.

Since GH and PRL affect a number of organs in the body that are known to change in  $\mu g$ , the rationale for evaluating the biological potencies of these hormones is obvious. Representative activities and bioassays for GH and PRL are shown in Appendix Table-2.

Why Spaceflight? The previous pituitary data argument. Rats and rat pituitary cells have been "flown" under both  $\mu g$  and  $\mu g$ -mimicked conditions (Appendix-Table 3). Hymer, the PI of the experiment to be done on the IML-2 mission, has also been PI or Co-I on each of these previous 5 spaceflights involving pituitary tissue. The

results of these missions have been reviewed recently (Hymer, W. C., K. Shellenberger and R. Grindeland. 1992. Pituitary Cells in Space. Adv. Space Res. In press.) and described individually in several refereed journal articles. Taken together, the results demonstrate repeatable and statistically significant changes in the pituitary GH and PRL cell systems. (Appendix Figs. 1,2, Tables 4,5.) They therefore provide one experimental rationale for the IML-2 science. The reader will note that virtually all of the data shown in Appendix Figs. 1 and 2 and Tables 4 and 5 were obtained on pituitary cells prepared from spaceflown rats postflight. A successful IML-2 experiment requires that similar changes occur within spaceflown pituitary cells themselves. On the 8 day mission of STS-46 (July, 1992) the PI conducted an experiment (sponsored by NASA Code SB) which flew 165 glass vials, each containing  $2 \times 10^5$  rat pituitary cells. Test variables included culture medium, GH cell subpopulations, GH cell position, GH cell sensitivity to hydrocortisone and finally sensitivity of flight cells to hypothalamic peptides (GRF) postflight. Each of these variables was assessed relative to the biologic potency of secreted GH and PRL during flight. Many of the > 2000 samples generated are in assay at the time of writing this science requirements document. However, key findings to date are summarized in Appendix Table 6. They clearly show that  $\mu\text{g}$ -induced changes occur within the pituitary GH/PRL cell and obviously strongly support the idea that the changes seen in pituitaries of spaceflown rats are explicable by changes within the pituitary cells themselves. This is a critically important result because it also establishes the first requirement of a successful IML-2 experiment, viz that in order to test for mechanisms

which may explain our previous  $\mu\text{g}$  results, similar changes must occur within the cells themselves.

Why Spaceflight? The Data Base Argument. Intracellular biological processes (and mechanisms controlling them) are often very different in the  $\mu\text{g}$  environment. Dickson (ASGSB Bulletin 4(2) 151, 1991) has conveniently summarized the results of over 500 spaceflight experiments (beginning in 1960) in which cells have been exposed to  $\mu\text{g}$ . There is no question that many different bacterial and mammalian cell types "sense"  $\mu\text{g}$  and respond differently. Going to space therefore provides a laboratory environment that will eventually help define the role that gravity plays in driving these processes on earth.

Why spaceflight? The continuous flow electrophoresis (CFE) process per se argument. While CFE has been used to separate cells and organelles in the past (examples; Hannig et al., Electrophoresis 11, 600, 1990; Heidrich et al., Methods in Enzymology 171, 513, 1989; Clifton et al., Electrophoresis 11, 913, 1990), there is agreement that gravity limits resolution of separation because of: a) sedimentation during exposure to the electric field, b) zone sedimentation due to density gradients caused by diffusion boundaries (droplet effect), and c) convection due to heating of the suspending medium by the electric current. The interesting experiment by Hannig et al. (Electrophoresis, 1990) clearly establishes the fact that in the case of cell separation resolution is better in  $\mu\text{g}$ . Previous results on the McDonnell Douglas CFES

device also demonstrate the  $\mu\text{g}$  advantage in terms of protein concentration. Finally, the PI conducted a pituitary cell separation experiment using CFES on STS-8. The result demonstrated cell separation in  $\mu\text{g}$  but low yields required substantial cell pooling. This, in turn, did not allow us to make statements concerning enhanced resolution in space. (See Appendix Fig. 4A, 4B and 4C for data which substantiate these claims).

It is well known that many harmful effects (e.g. electroosmosis, sedimentation, Joule heating, natural convection and electrohydrodynamics) create problems for separation of biologics by CFE. In the recent theoretical/experimental study by Clifton et al (Electrophoresis, 11, 913, 1990) one finds: "The aim of this study was to provide some quantitative information dealing with the influence of the main parameters, such as chamber geometry and operating conditions, on the performance of continuous flow electrophoresis. A model was presented that can determine the conditions under which any instability due to natural convection may occur and disturb the flow pattern inside the chamber. It was found that the existence of instabilities is directly correlated to a dimensionless group,  $Gr/Re$ , which is strongly dependent on the chamber geometry, i.e., its thickness, as well as on the operating conditions, i.e. the product  $E\tau$  of the electric field strength times the residence time. A model was also developed which distributes the products in a cross section near the outlet of the electrophoretic chamber, under such conditions that no instability disturbs the flow. This model takes account of diffusion, electroosmosis, migration and three-dimensional flow map of the carrier fluid, assuming that

electrohydrodynamics is negligible. The results that come from that model were compared with experimental ones obtained for various chamber geometries, operating conditions, and proteins. It was found that there is good agreement between theoretical and experimental results, even when working with a mixture of several kinds of proteins (hemoglobin, bovine serum albumin and cytochrome C)." Clifton concludes: "If instabilities due to natural convection are to be feared, there are two possible solutions: (i) operate in microgravity, where the conditions for flow stability are easier to fulfill; (ii) operate on earth with a thinner chamber so as to keep a stable flow pattern, but, in this case, only a partial separation can be obtained."

In conclusion, the variables associated with CFE at 1g and  $\mu$ g include not only 1) confounding forces within the electrophoresis chamber (see above) but also 2) the biologic complexities associated with protein molecules  $\rightarrow$  cells themselves. Indeed, issues relating to interactions between sample and carrier buffer (conductivity mismatches, electrohydrodynamics) are the primary focus of the other electrophoresis experiment being done on the IML-2 mission (R. Snyder, PI; Ramses hardware). We expect that the electrophoresis data obtained using model particles (polystyrene latex), coupled with data using relevant biological particles (pituitary cells/subcellular organelles), will permit a more thorough understanding of the advantages of CFE in space.



### 1.3 Value of Knowledge to Scientific Field

Snyder states "increased product yield can be as important as high resolution for many biological materials and increased sample concentration is the main mechanism for providing increased throughput in space"; (see Science Requirements Document, IML-2). In the field of biotechnology however, existing cell separation methods (flow cytometry, density gradient centrifugation, elutriation) on earth are sometimes insufficient to obtain minority cell subpopulations of high scientific interest (e.g. cells of the immune system) or organelles/proteins of similar importance. Results of cell → protein separations by CFE on earth are relatively unknown in the science community even though some laboratories have been "in the game" for > 15 years. The fact that CFE equipment is relatively expensive has hindered its development. In space the situation is worse although some preliminary results have been encouraging (Appendix Fig. 4A). It is important to note that several countries have taken serious interest in CFE in  $\mu\text{g}$ . Japan flew FFEU for the first time on the SL-J mission in 1992; Germany has flown its unit on sounding rockets; France will fly its RAMSES unit on IML-2 and the Russians have flown their unit on MIR many times. At Penn State University, the Center for Cell Research is building its flight unit for use on several SpaceHab missions beginning in 1994.

### 1.4 Justification of the Need for the Space Environment

In Section 1.2 three arguments are offered as justification for spaceflight. These are:

- the previous pituitary data argument
- the data base argument
- the CFE process per se argument

It is not necessary to repeat these arguments again in this section. However, an additional justification argument, somewhat more general in nature, now follows.

The PI has completed pituitary based experiments on 6 Code S-sponsored spaceflights and 2 Code C-sponsored spaceflights. The IML-2 experiment has evolved naturally from these previous missions. Only a few investigators have had the opportunity to participate in as many experiments. Since the focus of these previous efforts has been varied (i.e. electrophoresis, cell culture, rats), and since different experimental approaches are probably required to eventually understand how the pituitary gland "works" (Appendix Table 1), our repeatable findings establish a pituitary  $\mu\text{g}$  data base that pertains directly to the TX-101 experiment. With that knowledge we can now begin to ask mechanistic questions about changes in the GH/PRL cell systems in  $\mu\text{g}$ . We no longer have to phrase the experimental question in the form "... what is the effect of  $\mu\text{g}$  exposure on ...?" To the scientist unfamiliar with the peculiarities and difficulties associated with  $\mu\text{g}$  experimentation, the argument may not seem particularly strong. However, an experiment such as TX-101, which calls for numerous, complex sets of tasks which include transfer of samples from one piece of hardware to another, becomes technically demanding. As a demonstration experiment for future  $\mu\text{g}$  research, the potential benefits become greater too. A successful experiment will help meet the following goals.

## EXPERIMENTAL GOALS

- establish the kinetics of release of bioactive GH/PRL in  $\mu\text{g}$
- establish electrophoretic mobilities of the pituitary cells in space after 5 mission days; a time when bioactive hormone output is probably changing as well
- establish electrophoretic mobilities of pituitary organelles in  $\mu\text{g}$  prepared from  $\mu\text{g}$ -exposed cells
- demonstrate enhanced resolution of separation CFE using process on two different biological particles (granules/ cells)
- Coupled technology demonstration using Japanese hardware

## IMPLICATIONS

- if changes are more significant in media from 3rd culture change than 1st, then countermeasure strategy may be different
- CFE would be useful tool for indicator of cellular dysfunction in  $\mu\text{g}$
- CFE would be useful tool for preparation of cell organelles for eventual study on earth or in space
- define utility of space CFE in future biotechnology experiments
- an example for future biotechnology research on SSF

## BACKGROUND

### 2.1 Description of Scientific Field to Which Experiment Belongs

Modern biology uses techniques and approaches which are usually classified as belonging to the fields of molecular and/or cellular biology. More traditional designations of biological sciences; e.g. biochemistry, physiology, etc. use techniques which cut across these molecular and cell biology fields. To make matters slightly more confusing, the field of biotechnology has evolved from its gene-splicing origins to investigations which use combinations of state of art technologies to obtain answers to biological problems which can have both basic and practical applications as end goals.

The TX-101 experiment uses two techniques, viz. cell culture and continuous flow electrophoresis to determine 1) mechanisms by which  $\mu\text{g}$  affects pituitary cell function and 2) efficiency of operation in this environment. It can be argued that the former goal is basic in character; the later is more applied (i.e. biotechnological).

### 2.2 Current Applications for Research in Field

Heterogeneity is the hallmark of biological systems. It occurs at all levels; from molecules (isoforms) to subpopulations of cells which reside in tissues. Moreover, it is common among many organ systems (e.g endocrine, immune, musculoskeletal, etc)--many of which are affected by  $\mu\text{g}$ . Accordingly, the TX-101 experiment has widespread applications for this scientific field (section 2.1) on earth and in space.

### 2.3 Brief Historical Account of Prior Research

- The PI has done pituitary research for over 30 years (see resume).
- The PI has participated in 8 previous spaceflights, each involving the pituitary gland.

### 2.4 Current Research

In the PI's laboratory, current research projects involve:

- study of pituitary-immune interrelationships, especially the role that prolactin plays in modulating function of T-lymphocytes. Special emphasis is placed on importance of these findings to breast cancer. Dr. Andrea Mastro, Co-I of the TX-101 experiment, is also Co-I of this NIH-sponsored grant.
- postflight analyses of the PHCF experiment (Pituitary Hormone Cell Function) done on the STS-46 flight, July 1992.
- human growth hormone studies in relation to activities sponsored by NASA's Office of Commercial Programs via a commercial development of space grant to W. C. Hymer (PI) at Penn State's Center for Cell Research.

### 2.5 Relationship of Proposed Experiment to Scientific Field

See Section 2.1

## 2.6 Anticipated Advance in State of the Art

The results will help determine the usefulness of separation technology by continuous flow electrophoresis in space when applied to biologic samples that have medical relevance for astronauts and people on earth.

# **JUSTIFICATION FOR CONDUCTING THE EXPERIMENT IN SPACE**

## 3.1 Limitations of Ground-based Testing

Some of the complex forces controlling separation efficiency of biological samples in  $\mu g$  are reduced. Thus not only can one begin to sort out and understand the variables which confound separations on earth, but one can also begin to determine the unique advantages of applying electrophoresis technology in space (see Section 1.2). Our preflight data collected thus far (see Section 4.5) on CFES/FFEU hardware demonstrate the limitations of these devices on earth. With specific reference to pituitary cells/granules, preflight experiments show that cell sedimentation leads to poor cell recoveries which in turn require sample pooling, thus diminishing quality of the separation.

## 3.2 Limitations of Drop Towers

The residence time for a CFE process is several minutes; the drop tower is not a valid test vehicle.

### 3.3 Limitations of Testing in Aircraft

Not a valid test vehicle for the identical reason given in 3.2.

### 3.4 Need for Accommodations in the Shuttle

The time, environmental and power requirements for the experiments in TX-101 can only be met in the Space Shuttle.

### 3.5 Limitations of Mathematical Modeling

There are no models that predict electrophoretic behavior of pituitary cells/organelles in a way that predicts meaningful separations in terms of particle function (i.e. the heterogeneity issue, section 2.2). General models recently published by Albrecht-Brehler and Kondepudi (ASGSB Bulletin 4(2) 119, July 1991) address the issue of ability of biologic particles in the cell → organelle size range to "sense"  $\mu\text{g}$ . Results of the TX-101 experiment may be interpretable in terms of these models; as such they may provide data to establish to utility of these models in future missions.

In a similar vein, the results from TX-101 may be applicable and tested against the mathematical models being developed for CFE by Dr. Snyder on his IML-2 experiment. Dr. Snyder is also Co-I of TX-101.

### 3.6 Limitations of Other Modeling Approaches

Not applicable.

## EXPERIMENT DETAILS

### 4.1 Experiment Procedures to be Used

There are 3 parts to the experiment and each part interrelates with the other two.

#### PART I. PITUITARY CELL CULTURE IN JAPANESE CELL CULTURE KIT (CCK).

##### Background Rationale

While the techniques associated with pituitary cell culture are well established (> 20,000 publications have appeared in the literature between 1973-1991), the pituitary cell system is complex, heterogeneous and incompletely understood. It has been used primarily to test how hormones isolated from brain tissue regulate the synthesis and release of pituitary hormones.

The PI's interest in the use of pituitary cell culture technology stems from two facts regarding biological heterogeneity in this system; first, that at least two subpopulations of both GH and PRL cells are contained in the rat pituitary and second, that the biological (B) and immunological (I) activities of GH and PRL secreted from these cells are not necessarily equal (i.e. B/I # 1). An understanding of the functional significance of this heterogeneity, as well as mechanisms involved, is relevant to both the medical and basic science community.



In section 1.2, the rationale and preliminary results (Appendix Table 6) from the STS-46 pituitary cell culture experiment were considered. There is now no longer any doubt that pituitary cells, by themselves, function differently in  $\mu g$ . It seems possible that altered bioactivity of GH/PRL molecules in  $\mu g$  may contribute to some of the better known defects in target tissues in  $\mu g$ , viz the musculoskeletal and immune systems.

#### Hypotheses Under Test

- The changes observed in previous spaceflight missions involving rats are attributable to intracellular mechanisms originating within the pituitary gland. These changes do not depend upon blood-mediated factors or events.
- GH and PRL cells in the Japanese culture hardware will release less bioactive GH and PRL in microgravity.
- Bioactive hormone levels will continue to change in  $\mu g$ ; this change will be reflected in culture media collected at different times in flight
- GH and PRL cells in the Japanese culture hardware will not be able to release as much bioactive GH and PRL on return to Earth as control cells which have never been in space. That is, their "recovery" kinetics will be different from control.

### Preflight Preparation

At L-36 hrs. 100 young (200 g) adult male SPF rats are killed and  $3 \times 10^8$  sterile anterior pituitary cells are prepared by trypsinization.

- $\sim 70 \times 10^6$  cells are seeded into each of two cell culture hardware units (CCK), each containing 20 ml of medium ( $\alpha$  MEM + 5% calf serum + antibiotics + 25mM Hepes, pH 7.4).
- One of the cell culture units is kept on the ground (Hanger L) @ 37°C; the other is turned over to STS (37°C) at L-32 hrs. The later CCK is used for Part I.
- The remaining cells ( $\sim 1.9 \times 10^8$ ) are distributed equally into two additional cell culture units ( $0.95 \times 10^8$ /chamber) in medium (above) and placed onboard STS. These two chambers will serve as the experimental material for Parts II and III respectively.

## PART II: PITUITARY CELL SEPARATION IN MICROGRAVITY

### Background Rationale

The PI has over 20 years experience in the field of pituitary cell separation. Techniques developed in my laboratory for pituitary tissue include separations which are based on differences in a) cell size (velocity sedimentation at unit gravity); b) cell density (density gradient centrifugation); c) cytoplasmic secretory granule content (laser light scatter) and d) cell surface charge (continuous flow electrophoresis).

Results from numerous studies document the usefulness of these cell separation techniques for the study of functional heterogeneity of specific hormone-producing cell types. For example, a subpopulation of GH cells which release hormone that is rich in bioactivity (i.e. has a high B/I activity ratio) can be separated from a subpopulation which releases GH with a low B/I activity ratio by either density centrifugation or continuous flow electrophoresis. Mechanism(s) which account for these consistent subpopulation differences in both GH and PRL cell subtypes are unknown.

A continuous flow electrophoresis device (McDonnell Douglas Corporation) was used on the STS-8 mission (1983) in an effort to determine the role that gravity might play in earth based separations. We determined that the quality of the cell separations were of the same order on the earth as they were in space. This result was important in the sense that it established that electrophoretic separations achieved in spaceflight were based on differences in surface charge and not (as we seriously considered) due to "fall back" of GH cells in the electrophoresis column during earth based runs. Unfortunately, technical problems encountered during the experiment prohibited answers relating to issues of subpopulation differences, B/I activities and cell-cell communication differences. These can now be elaborated in the IML-2 mission.

#### Hypotheses Under Test

- The efficiency of the cell separation device (FFEU) in space will be superior to that on the ground (as evidenced by the higher purity of GH

and PRL cell containing fractions). High quality cell separations can be achieved in spaceflight under conditions where cells are exposed to low conductivity buffers for <60 minutes over the course of a 13 day mission.

- The most electrophoretically mobile (anodal) GH cell subpopulations will be most negatively affected in terms of abilities to produce bioactive GH.
- The electrophoretic mobility of spaceflown GH cells will be altered because of hormone differences on the cell surface.

### PART III: PITUITARY SECRETORY GRANULE SEPARATION IN MICROGRAVITY

#### Background

The PI was among the first (1963) to show that GH-containing granules could be isolated from rat pituitary tissue and that, after injection into hypophysectomized rats, these particles were able to promote body growth. Results of subsequent studies by many investigators show that:

- the intragranular pH is 5.
- the H<sub>2</sub>O content is low (1  $\mu$ l)
- divalent cations are copackaged
- the hormone is probably packaged in a crystalline state

- various pumps ( $H^+$ ,  $Ca^{+2}$ ) are probably contained in the granule membrane

Recent work in the PI's laboratory show that:

- $Zn^{+2}$  (0.2mM) stabilizes immunoactive GH in the particle
- GH extracted from cells with large numbers of secretory granules show (by Western blotting) oligomeric forms of monomeric (22K) GH whereas GH extracted from cells with fewer numbers of secretory granules do not show aggregated hormone
- Lysates (containing granules) run on the McDonnell Douglas continuous flow electrophoresis system show heterogeneity in electrophoretic mobility.

As indicated in Appendix Table 4, GH cells prepared from flight rats are consistently more intensely stained with fluoresceinated antibody to GH than corresponding cells from ground control animals. Since a majority of intracellular hormone is stored in the secretory granule, we believe the result can be attributed (in part) to different packing of GH molecules within the granule in space.

#### Hypotheses Under Test

- Cross-linking of intragranular GH monomers via S-S bridging will be less efficient in space. This, in turn, will expose more epitopes and decrease bioactivity of the GH.

- Organelles prepared in  $\mu\text{g}$  from space flown cells will have different electrophoretic mobilities than ground based controls.

#### 4.2 Measurements required

The statements and ideas presented in this section are based on two years of preflight research and extensive discussions and planning with several individuals within NASDA (primarily Drs. Nagoaka, Mukai, Kumei and Kobayashi). These statements and ideas are also a consequence of data collected from samples electrophoresed on Japanese flight hardware. These statements represent the PI's current understanding of events that will occur on the IML-2 mission. They are not to be considered as being "cast in stone." Material presented below will be cross-referenced with that in sections 4.5 and 4.6.

### PART I - MEASUREMENTS

#### Flight

- on every 3rd mission day (3 times total), the 20 ml of culture fluid will be removed from the chamber and transferred to a storage tube (stored frozen on Shuttle).
- fresh medium will be injected into the chamber on every 3rd mission day.
- A photographic record of the cells before one or more medium changes is required.

## PART II - MEASUREMENTS

- On the 5th mission day the medium in CCK-#2 is removed for storage and cells are washed in electrophoresis buffer [EB (5.0 mM Hepes) 30 mM Glycine, 0.2 mM KAc, 0.3 mM MgCl<sub>2</sub>, 0.03 mM CaCl<sub>2</sub>, 220 mM Glycerol, 44 mM Sucrose; 0.2 mM Zinc Chloride, 0.1% BSA, pH 7.2; conductivity 250  $\mu$ mhos; Osmolarity; 315 mOsm].
- Cells are removed from the polycarbonate substrate by trypsinization and then washed again in electrophoresis buffer containing DNase. Cell-washing is accomplished utilizing the unique design of the CCK.
- Cells are electrophoresed @ 150V (25 v/cm) at flow of 3 cm/min. The UV detector captures light scatter signals as cells leave the chamber; a resulting "scattergram" is viewed by the astronaut in real time. This determines the position of the 30 collection tubes within the 60 tube template. Each collection tube is charged with serum-containing medium prior to launch to enable post electrophoresis culture in  $\mu$ g in serum-containing medium.
- Cells in the 30 collection tubes are cultured @ 37°C for the remainder of the flight (except for ~ the last 24 hours when temperature is ambient).

### PART III - MEASUREMENTS

- on the 5th mission day the medium in CCK-#3 is removed for storage prior to cell lysis and electrophoresis.
- cells are lysed in H<sub>2</sub>O containing 0.2 mM Zn<sup>+2</sup> (~5') and the lysate concentrated by a mechanism TBD (dialysis/filtration are options at this point).
- the lysate is electrophoresed on FFEU (150 V; 25V/cm; 3 cm/min) and the separation monitored at chamber outlet scattergram technology described using in Part II measurement (above).

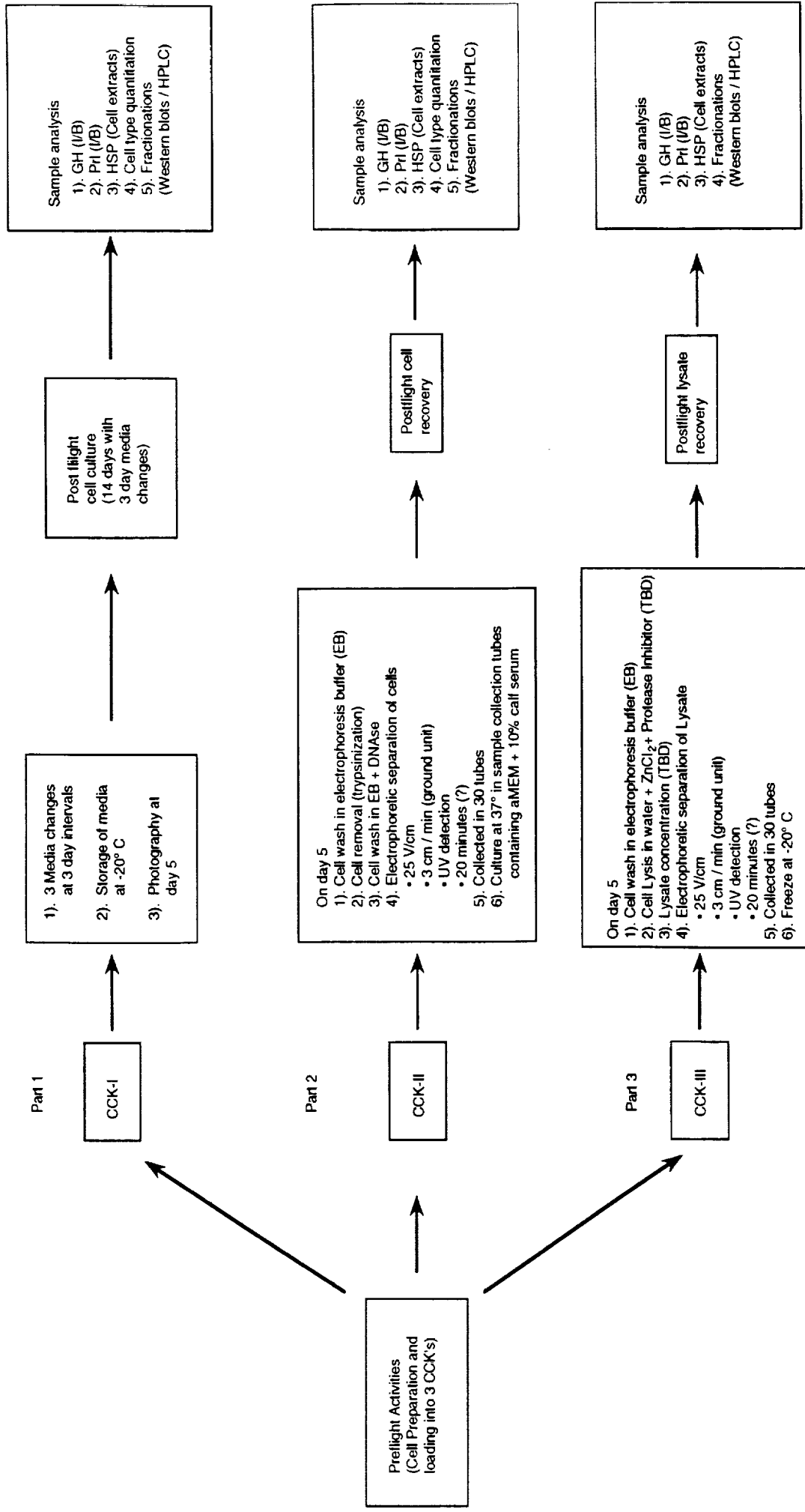
#### 4.3 Test Plan Including Ground Characterization of Flight Hardware

A functional flow diagram of the TX-101 experiment detailed in 4.1 is given (see next page).

The data in Appendix-Figures 5-23 present results of experiments on the FFEU hardware that reproduce procedures in Parts I, II, and III. These data (to be discussed at Science Review 1/29/93) show that the new version (12/92) of the CCK will be suitable for pituitary cells:

- pituitary cells can be: cultured for 5 days; recovered from the flask; electrophoresed through the FFEU and finally cultured post-electrophoresis for several days [this follows the entire procedural sequence for Part II]





EB composition:

- 5 mM HEPES
- 30 mM Glycine
- 0.2 mM Potassium Acetate
- 0.3 mM Magnesium Chloride
- 0.03 mM Calcium Chloride
- 220 mM Glycerol
- 44 mM Sucrose
- 0.2 mM Zinc Chloride
- 0.1% BSA

pH 7.2 Conductivity 250 µmhos 315 mOSM

- pituitary cell lysates can be prepared from cultured pituitary cells and subsequently electrophoresed on FFEU. [This follows the entire procedural sequence for Part III].
- all of the experiments done on Japanese hardware can be repeated using hardware at Penn State (i.e. CFES).

#### 4.4 Specific Analyses Required

See Section 5.8 and Section 4.6.

#### 4.5 Preflight Experiment Planned

Ground operations will in general follow prelaunch procedures developed for the PHCF experiment for the STS-46 mission (7/92). This includes:

- maintenance of SPF young adult (50 days old) male rats at KSC
- facilities for animal sacrifice
- facilities for preparation of living, sterile pituitary cells
- charging 4-6 CCK with pituitary cells (37°C)
- handover of 3 CCK units (37°C) to appropriate personnel (L-20)
- maintenance of remaining CCK units at temperature identical to those CCK's on STS (temperature monitoring and recording required throughout entire mission).

Experimental trials in 1993 will continue to be done on a combination of the following electrophoresis units:

- 1) CFES at Penn State (1.5mm thick chamber)
- 2) a FFEU prototype which is being built in Japan and is to be installed at Penn State 2/93 (chamber is 1 mm thick)
- 3) FFEU flight hardware (2 additional runs in Japan in 1993; 4 mm thick chamber).

Experimental trials in 1993 will also continue to use several CCK units which are currently at Penn State. The post-electrophoresis cell/lysate collection tubes will come to Penn State in 1993, as well as an yet undefined apparatus for lysate concentration prior to CFE. As of 1/29/93, details of the arrangements for getting the FFEU prototype; cell collection tubes and lysate concentrators to Penn State are currently in negotiation between the TX-101 investigator team and NASDA.

#### 4.6 Postflight Data Handling and Analysis

The 3 CCK's, 30 collection tubes containing live cells (temperature  $22 \pm 1^\circ\text{C}$ ) and 30 collection tubes containing frozen samples are removed from Shuttle and transported to Hanger L. The following experimental manipulations are then required:

- cells in CCK from Part I will continue to be cultured in the CCK at  $37^\circ\text{C}$  in Hanger L for an additional 7-14 days. Media will be removed at 3 day intervals and periodic cell photography will also be done.
- cells in each of the 30 collection tubes from Part II will be centrifuged (1200 RPM); the supernatant fractions will be frozen for subsequent analysis at Penn State; cells in the pellets will be prepared for

morphological analysis (immunocytochemistry) and immunofluorescence by flow cytometry.

- frozen material in the 30 collection tubes from Part II will be shipped to Penn State for analysis.

#### Postflight hormone assays

##### GH

Enzyme immunoassay (I)

tibial line bioassay (B)

IM-9 lymphocyte bioassay (B)

##### PRL

enzyme immunoassay (I)

splenocyte mitogenic assay (B)

Nb-2 mitogenic assay (B)

#### Other Analyses

Heat shock protein/chaperone on cell extracts.

Morphology (flow cytometry, immunocytochemistry).

Western Blots/HPLC for hormone variant analyses.

#### 4.7 Mathematical Models Used

None are anticipated, although some data will be interpreted within the context of Dr. Snyder's data on RAMSES on this same mission. Dr. Snyder is a co-investigator on the TX-101 experiment.

#### 4.8 Application of Results

See Section 1.1 and 1.2.

## **EXPERIMENTAL REQUIREMENTS**

#### 5.1 Experiment Sample Requirements

The samples will be rat pituitary cells in 3 CCK's. Hardware for this experiment will be provided via NASDA. See Section 4.1 - 4.3 for detailed requirements.

#### 5.2 Atmospheric Requirements

5.2.1 Pressure. Requirement will be the same as for FFEU experiment.

5.2.2 Gas Composition. Atmospheric requirements are compatible with normal gas composition of Shuttle

5.2.3 Humidity. Requirement will be same as for FFEU experiment

5.2.4 Vacuum. No requirements

#### 5.3 Temperature Control and Measurement

Temperature control within locker holding CCK's must be  $\pm 0.1^{\circ}\text{C}$  and temperature recording must be continuous and tracing provided to PI postflight.

Temperature control and monitoring within FFEU will be done by NASDA.

Temperature in SpaceLab environment must be monitored and recorded. Pituitary cells are sensitive to increased temperature (above  $37^{\circ}\text{C}$ ).

Cells collected postflight electrophoresis (in 30 collection tubes) will be returned to locker and kept at  $37 \pm 0.1^\circ\text{C}$  until 10 hrs. prior to reentry when temperature will be reduced to  $22 \pm 1^\circ\text{C}$ . Constant recording of temperature is required for this phase as well. (The 10 hr. temperature switch is dependent upon NASDA requirements).

#### 5.4 Vibration Control and Measurement

Same as for FFEU hardware.

#### 5.5 Test Matrix

See sections 4.1-4.3.

#### 5.6 Imaging Requirements

Cells in CCK will be photographed as described in Section 4.1. Samples flowing through FFEU will be monitored by UV unit on FFEU and data recorded.

5.6.1 Photography. Astronaut takes pictures and changes film roles. Ten Fields of view should be taken through microscope at two magnifications.

5.6.2 Radiography. None

5.6.3 Television. None (?)

5.6.4 resolution. None

5.6.5 Frame rate. None

5.7 Electromagnetic limitations. None.

## 5.8 Astronaut Involvement

5.8.1 Astronaut Involvement

5.8.2 Activation of experiment

### Part I - CCK #1

1. Remove CCK from 37° incubator in locker at MD3.
2. Remove medium and stow in -20°C freezer.
3. Add fresh medium to CCK.
4. Repeat steps 1-3 on MD5 and 8(?).
5. Switch temperature of incubator to ambient on MD12.
6. Photograph cells in CCK #1 on MD5.

### Part II - CCK #2

1. Remove CCK #2 from 37° incubator in locker at MD5.
2. Remove medium and stow in -20°C freezer.
3. Wash cells in electrophoresis buffer - stow wash @-20°C.
4. Add trypsin medium to promote cell detachment. Check for efficiency of cell detachment by microscopic observation; continue incubation until completion (usually ~ 15 min.).
5. Remove trypsin medium; stow at -20°C.

6. Add fresh electrophoresis buffer to CCK (vol = 5 ml); remove cells into FFEU sample syringe.
7. Electrophorese cells.\*
  - 150V (25 v/cm)
  - 3 cm/min.
  - UV detector (scattergraph)
  - Total run time 20 min. (further discussions with NASDA required)
8. Collect 30 fractions\*\*.
9. Store 30 fractions in 37°C incubator.
10. Wash FFEU chamber with electrophoresis buffer.

### Part III - CCK #3\*

1. Remove CCK from 37°C incubator in locker after Part II electrophoresis run is completed (MD5)
2. Remove medium and stow at -20°C.
3. Wash cells in CCK in electrophoresis buffer; save electrophoresis buffer wash at -20°C.
4. Add cell lysis buffer to CCK and monitor cell lysis microscopically until complete.

\* These conditions are not yet defined precisely.

\*\* The scattergraph will define which 30 collection ports to use.



5. Concentrate lysate in concentration hardware (further discussions with NASDA required).
6. Electrophorese lysate.
  - 150V (25 v/cm)
  - 3 cm/min.
  - UV detector (scattergraph)
  - total run time 20 min (further discussions with NASDA required)
7. Collect 30 fractions.
8. Store 30 fraction tubes @ -20°C.

#### 5.9 Data Requirements

1. Voice communication with astronaut will be required during Parts II and III.
2. Photography of cells in CCK required
3. Temperature monitoring and recording of cells in 37° incubator and when cells are required to be kept at ambient temperature.
4. Monitor scattergram development for Parts II and III and keep photographic record.

#### 5.10 Telepresence and Telerobotics

None.

## 6.0 Principal Investigator's Requirements

### 6.1 Research Equipment

6.1.1 Preflight. Items of equipment required for the preparation of live, sterile rat anterior pituitary cells will be furnished from the PI's laboratory and by existing facilities in the laboratories at Hanger L. The procedures for the preparation of cells are identical to those used for the STS-46 mission in July, 1992 and can be submitted under separate cover.

6.1.2 Postflight Cell Culture. Incubators ( $37^{\circ} \pm 0.1^{\circ}\text{C}$ ) will be required at Hanger L for 14 days in order to complete Part I. Cells in collection tubes from Part II will be processed at Hanger L before being shipped to Penn State for analysis. Requirements for this step include access to clinical centrifuge, refrigerator and phase microscope. Final sample processing will be at Penn State. Samples collected from parts I, II, and III which are stored frozen on Shuttle will be kept in frozen state until analysis at Penn State. Samples from other parts I, II, III which were at  $37^{\circ}$ /or ambient will be maintained at  $22 \pm 1.0^{\circ}\text{C}$  between time of landing and processing in Hanger L.

### 6.2 Apparatus Design Assistance

The PI is working closely with Dr. Snyder (MSFC) and Dr. Nagoaka (NASDA) to ensure that a prototype FFEU reaches Penn State soon after the science review for

TX-101 on January 29, 1993 in Huntsville. Placement of this item at Penn State is absolutely critical for eventual experiment success.

Any further delays will begin to impact negatively on the success of this goal.

### 6.3 Consultation

None; but note that the PI team interacts closely with NASDA and MSFC teams.

### 6.4 Grant

A 2.7 year proposal to accomplish TX-101 was sent to NASA Headquarters from Penn State in December 1992. Without full funding this experiment will not be possible.

### 6.5 Services

None, other than has been specified and detailed throughout the document.

### 7.0 Other Requirements

It will be necessary to do ground-based control experiments post flight in order to meet experimental objectives/hypothesis testing. This must be done post flight because conditions surrounding the actual flight experiment must be faithfully mimicked (e.g. timing of the execution of the numerous experimental subtopics;

temperature; etc). These postflight ground control trials will therefore be asynchronous and primarily involve TX-101 Parts II and III. Since Part I is done entirely in CCK's, it may be possible to do synchronous controls and our experimental scheme provides for this opportunity; however, a successful effort will be dependant upon accurate temperature downlinks during the course of the flight. At the time of writing this document the PI is uncertain as to the feasibility of this requirement.

It will be necessary to repeat Parts II and III using the actual flight unit (FFEU); ideally this should be done in the Hanger L laboratories at KSC postflight. Since the thickness of the chamber of the flight unit is 4 mm, it will not be possible to flow cells/particles without significant flow distortions. Indeed, this is part of the justification (and advantage) for spaceflight electrophoresis. The thickness of the FFEU model that the PI team used in Japan on 11/92 to generate the data presented in the Appendix was 1 mm. Postflight trials will also be done in the PI's laboratory at Penn State using the FFEU prototype (chamber thickness 1mm).

TABLE 1 Methods Which Have Been Used to Study Mammalian Pituitary Cell Function on Earth

APPROACH	METHOD	ADVANTAGE(S)	DISADVANTAGE(S)
<i>In vivo</i>	Sample blood from animal and assay hormone levels	<ul style="list-style-type: none"> <li>Identifies dynamics of hormone secretion from pituitary</li> </ul>	<ul style="list-style-type: none"> <li>Cannot identify mechanisms of hormone synthesis/release</li> <li>Multiple sampling required to establish secretory dynamics</li> </ul>
<i>In vitro</i>	Incubate tissue slices	<ul style="list-style-type: none"> <li>Hormone secretion dynamics</li> <li>Study regional secretory dynamics</li> <li>Cell-cell contacts maintained</li> </ul>	<ul style="list-style-type: none"> <li>Long-term culture not possible</li> </ul>
	Cell culture	<ul style="list-style-type: none"> <li>Long-term studies of hormone synthesis/ secretion</li> <li>Morphologic studies (image analysis)</li> </ul>	<ul style="list-style-type: none"> <li>Cell-cell contacts lost</li> <li>Artifactual (e.g. serum)</li> </ul>
	Cell separation	<ul style="list-style-type: none"> <li>Identifies functional heterogeneity in subpopulations</li> </ul>	<ul style="list-style-type: none"> <li>As above</li> </ul>
<i>In vitro/in vivo</i>	Cell implantation into suitable host	<ul style="list-style-type: none"> <li>End organ response of hormone secreted from implant</li> </ul>	<ul style="list-style-type: none"> <li>Maintaining viability of cell implant &gt; 20 days</li> </ul>

Hymer, W.C., et al., 1992, Pituitary Cells in Space, Adv. Space Res., in press.

**TABLE 2** Representative Activities and Assays of Secreted Growth Hormone (GH) and Prolactin (PRL)

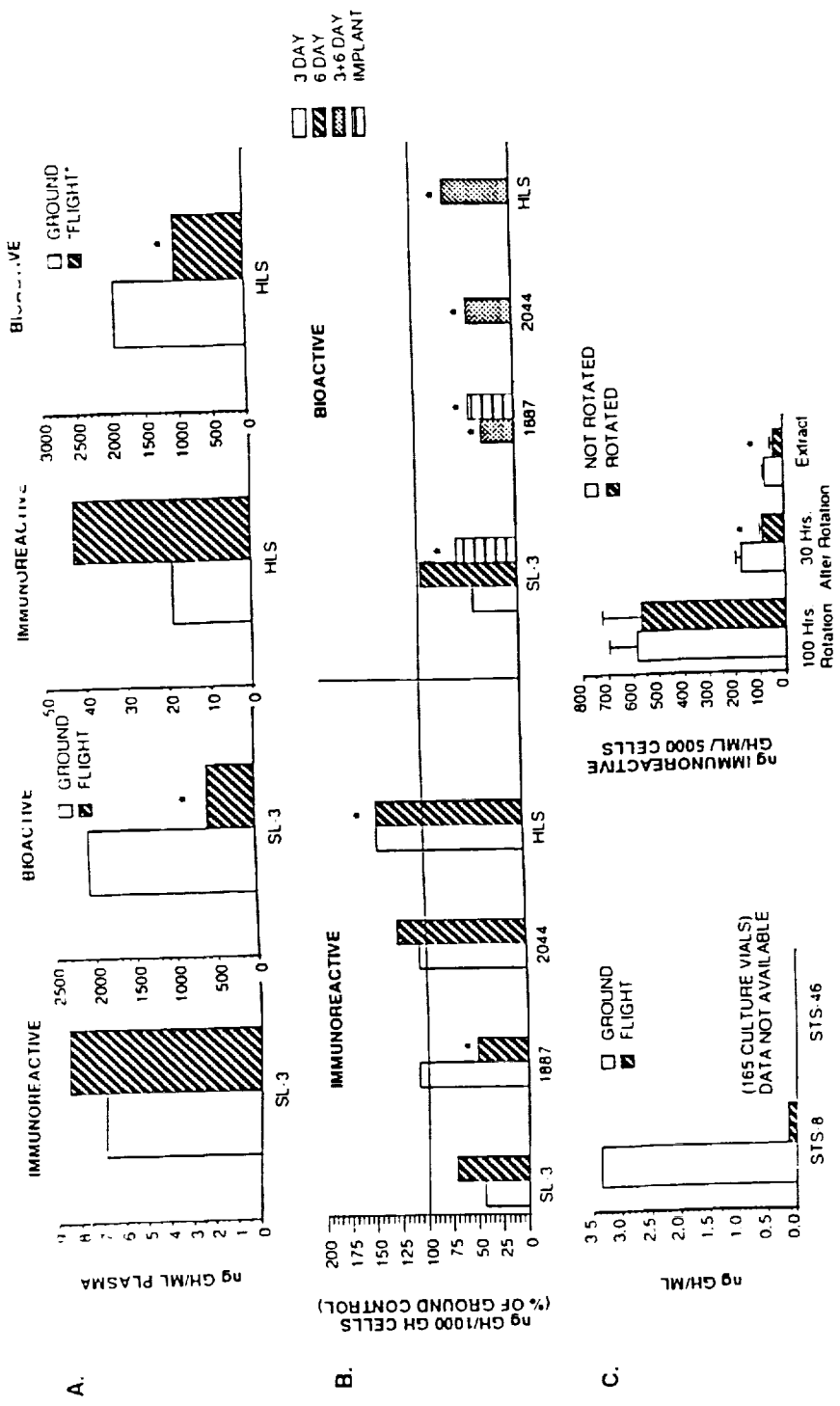
<b>A. Activities</b>	
<b>GH</b>	<b>Anabolic Agent:</b> ↑ Protein synthesis, muscle mass, bone growth <b>Lipolytic agent:</b> Promotes breakdown of fat <b>Diabetogenic:</b> Insulin antagonist
<b>PRL</b>	Milk synthesis Immune system modulator
<b>B. Assays</b>	
<b>GH</b>	Enzyme Immunoassay (EIA) [I] Radioimmunoassay (RIA) [I] Glucose metabolism in 3T3 cells [B] Tibial growth in hypophysectomized rat [B]
<b>PRL</b>	Enzyme immunoassay (EIA) [I] Radioimmunoassay (RIA) [I] Nb-2 lymphoma cell assay [B] IL-2R/mitogenesis assay in splenocytes from ovariectomized rats [B]

Hymer, W.C., et al., 1992, Pituitary Cells in Space, Adv. Space Res., in press.

TABLE 3 GH/PRL Secretion Data Collected from Spaceflight ( $\mu\text{G}$ ) or Mimicked Spaceflight ( $\text{m-}\mu\text{G}$ ) Experiments

SAMPLE	HORMONE ASSAYS	$\mu\text{G}$ EXPERIMENT	$\text{m-}\mu\text{G}$ EXPERIMENT
Rat plasma after spaceflight	iGH bGH	SL-3 (Fig. 1A)	HLS* (Fig. 1A)
Postflight pituitary cell culture media obtained from glands of rats flown previously in space	iGH bGH iPRL bPRL	SL-3 (Fig. 1B) Cosmos 1887 (Figs. 1B, 2A) Cosmos 2044 (Figs. 1B, 2A)	HLS (Figs. 1B, 2A)
Pituitary cells obtained from glands of rats flown previously in space and implanted into hypophysectomized rats on earth	bGH	SL-3 (Fig. 1B) Cosmos 1887 (Fig. 1B)	
Pituitary cells after spaceflight (including post-flight culture)	iGH bGH iPRL bPRL	STS-8 (Figs. 1C, 2B) STS-46 (Figs. 1C, 2B)	Clinostat (Fig. 1C)
Pituitary tissue after spaceflight	iGH		Clinostat*

\*HLS = Hindlimb suspension. \*Data not given in this report. Mission Length (days) - SL-3 (7); Cosmos 1887 (12.5) Cosmos 2044 (14); STS-8 (7); STS-46 (8).



**Fig. 1.** Release of growth hormone (GH) from animals and cells flown in space for 7-14 days or from rats subjected to hind limb suspension (HLS) for 14 days. These data are modified from /16, 17, 26, 27, 28/. **A.** Plasma levels of immunoreactive (I) and bioactive (B) GH in rats flown on the 7 day mission of SL-3. Note similar responses in HLS animals after 7 days of treatment. **B.** Release of iGH and bGH from cells of spaceflown or HLS rats (SL-3, 7 days; Cosmos 1887, 12.5 days; Cosmos 2044, 14 days; HLS, 14 days) during two successive 3 day postflight culture periods expressed as a percentage of ground-based controls. In two flight experiments cells from ground and flight animals were encapsulated in XM-50 hollow fibers, implanted into the cerebral ventricles of hypophysectomized rats and tibial epiphyseal plate widths evaluated 10-15 days post-implantation /24/. Data are expressed relative to number of GH cells in the sample as determined by flow cytometer. Levels of iGH ranged 25-143 ng/1000 GH cells; levels of bGH ranged 0.8-3.9  $\mu$ g/ml of combined 3 and 6 day media. Experimental design precluded statistical analysis of SL-3 data. **C. Left Panel.** Release of iGH from cells flown previously on the 7 day flight of STS-8. In this case primary rat pituitary cells were maintained in sealed, fluid-filled centrifuge tubes for flight; on return these cells were cultured for 6 days and media analyzed for released iGH. This preliminary experiment served as a precursor for the 8 day pituitary cell culture experiment flown on STS-46 in July 1992. **Right Panel.** Release of iGH from rat pituitary cells immediately after 100 hr. clinorotation at 1 RPM or 30 hrs. postrotation using the device initially used and described by Gruener and Hoeger /22, 23/. Results represent the average of 3 trials. \* $p < 0.05$ .



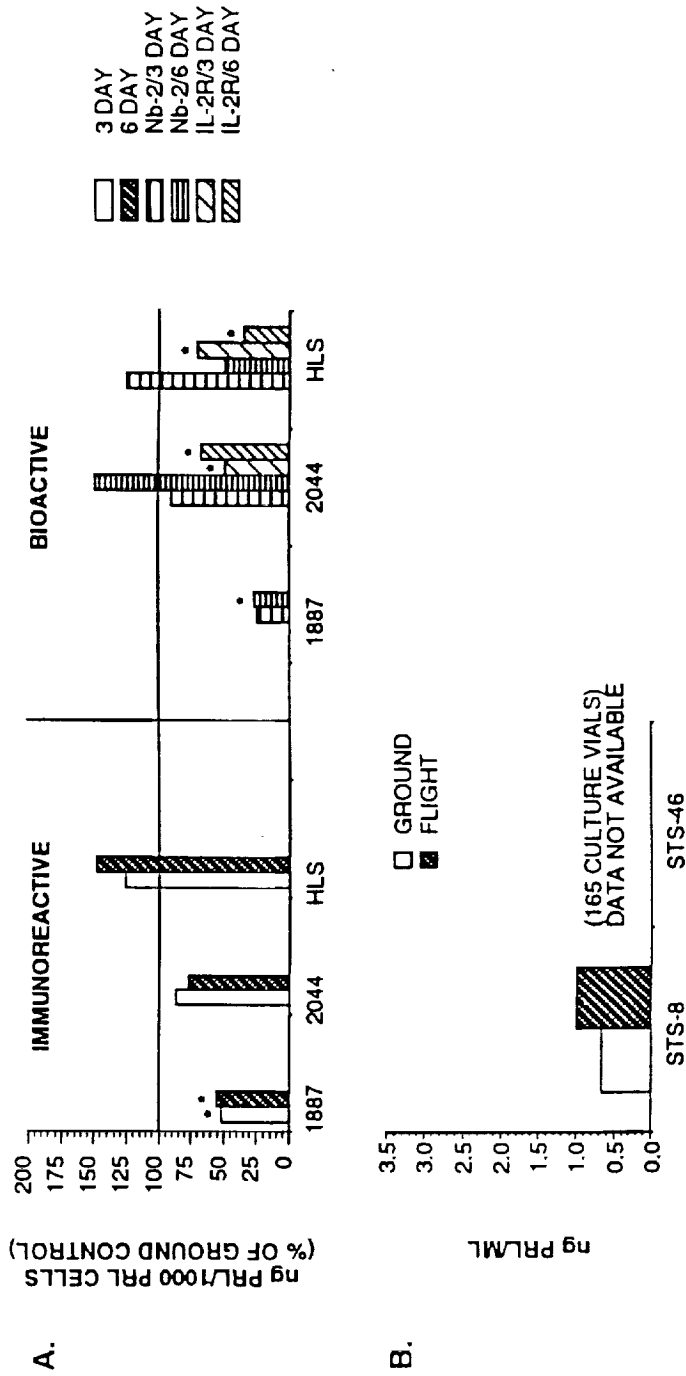


Fig. 2. Release of prolactin (PRL) from animals and cells flown in space for 7-14 days or from rats subjected to HLS for 14 days. See legend to Fig. 1 for details. A. bPRL measured by the Nb-2 bioassay of Tanaka *et al.* /11/ or the IL-2R bioassay of Mukherjee *et al.* /12/. Levels of iPRL ranged 10-80 ng/1000 PRL cells; those of bPRL ranged 6-48 ng/1000 PRL cells. B. Release of iPRL from cells flown previously on the 7 day flight of STS-8.

**TABLE 4. Cultured Somatotrophs from Suspended Rats Show Decreased Secretion of Bioassayable Growth Hormone and Decreased Response to Growth Hormone Releasing Factor.**

	Length of Suspension (days)	ng BGH/ ml Media
<u>nM GRF</u>	0	0.8
Control (6)	2400	4000
HLS (6)	1225	N.D.
Control (9)	1575	2813
HLS (9)	1200	1925
Control (8)	14	1525
HLS (8)	N.D.	1050
		5000
		1275

Numbers in parentheses represent numbers of animals. N.D. = not done. Data from reference 25.

Hyer, W.C., et. al., 1992. Pituitary Cells in Space, Adv. Space Res., in press.

TABLE 5 Intracellular Parameters of GH Cells from Spaceflight Rats or Rats Subjected to HLS

PARAMETER MEASURED IN FLIGHT CELLS	$\mu\text{G}/\text{m}-\mu\text{G}$ EXPERIMENT	% DIFFERENCE FROM GROUND CONTROL
Fluorescence intensity of GH staining	1887	+87
	2044	+82
	HLS	-2
GH cell size: forward angle light scatter	1887	+20
	2044	+25
	HLS	-3
GH cell granularity: perpendicular light scatter	1887	0
	2044	+11
	HLS	+3
Cytoplasmic area (%) occupied by GH	1887	+35*
	2044	+33*
	HLS	-7
<u>PRECULTURE POSTCULTURE</u>		
GH intracellular content (ng iGH/1000 GH cells)	SL-3	+56
	1887	-34
	2044	-
	HLS	-
GH intracellular content (ng bGH/1000 GH cells)	SL-3	+149*
	1887	-54*
	2044	-63*
	HLS	-63*
GH intracellular content (ng bGH/1000 GH cells)	SL-3	+104*
	1887	-20*
	2044	-76*
	HLS	-76*

\*3T3 cell bioassay results /14/. All other bioassays are from tibial line assay of Greenspan /13/. \*p < 0.05. Statistical analysis of flow cytometric data is not possible because measurements are made on a single pool of at least 10,000 cells. See text for additional details.

## RESULTS TO DATE FROM STS-46 MISSION (7/92)

- EXPERIMENT**
- 165 GLASS VIALS; 2 X 10<sup>5</sup> PITUITARY CELLS/VIAL IN 4 ml MEDIA
  - **VARIABLES**
    - CULTURE MEDIUM
    - GH CELL SUBPOPULATIONS
    - GH CELL GEOGRAPHY
    - GH CELL SENSITIVITY TO HYDROCORTISONE
    - SENSITIVITY OF GH CELLS TO GRF (2 X 10<sup>-9</sup>m) POST FLIGHT
  - **ASSAYS**
    - GH (I, B)
    - PRL (I, nb-2/MITOGEN)
    - CELL %, FLUORESCENCE

**RESULTS**

- NO CHANGE IN % CELL TYPES/VIABILITY > 90%
- ↓ RESPONSIVENESS TO HYDROCORTISONE
- UNRESPONSIVE TO GRF POSTFLIGHT
- SHUTDOWN IN B-GH FROM TYPE II CELLS

FIG. 4a

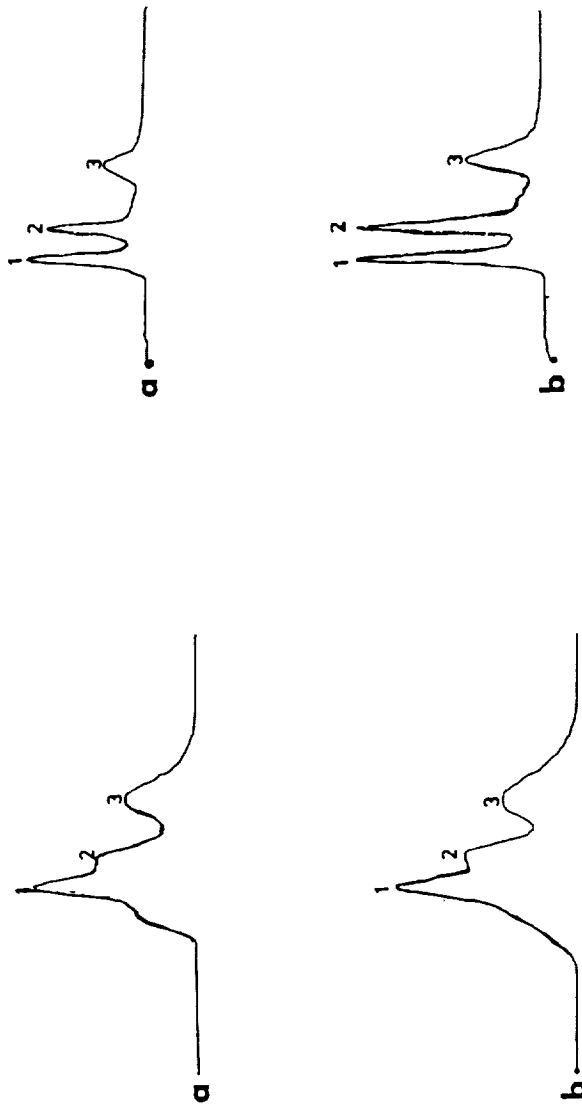


Figure 5. Electrophoretic separation of fixed erythrocytes from (1) rat, (2) guinea pig and (3) rabbit under 1 g-conditions. Recordings were taken at (a) 7 min, (b) 5.5 min and (c) 4 min before launch.

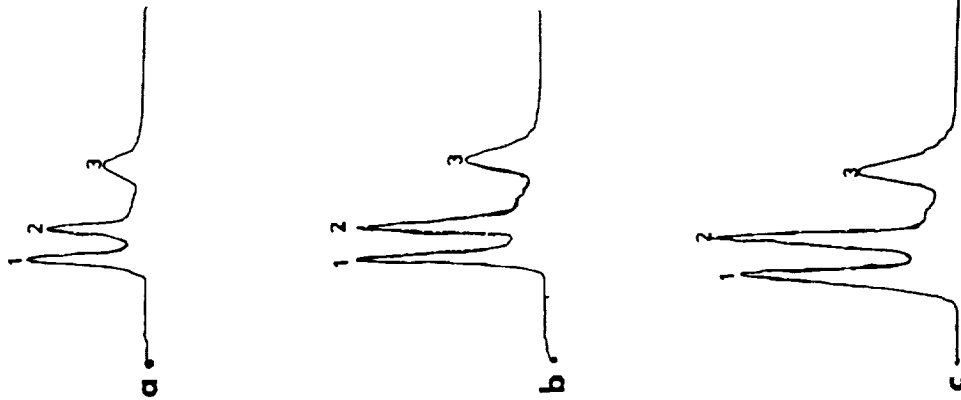


Figure 6. Electrophoretic separation under microgravity of fixed erythrocytes from (1) rat, (2) guinea pig and (3) rabbit. Recordings were taken at (a) 4 min, (b) 4.4 min and (c) 7.3 min after launch.

FIG. 4b

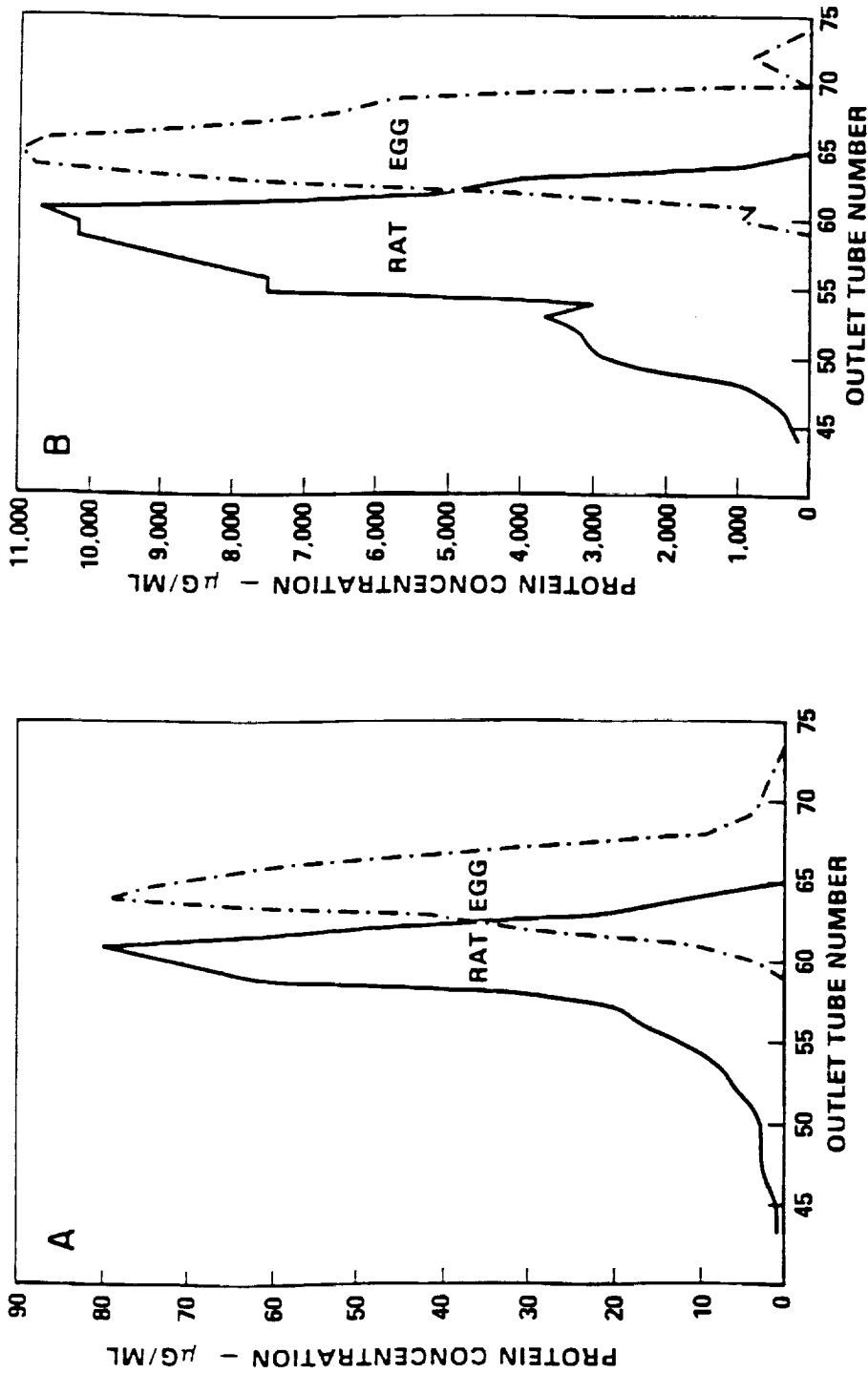


Fig. 2. Protein content of collected fractions after continuous flow electrophoresis in the laboratory (A) and in space flight (B) of a mixture of rat serum albumin (solid line) and ovalbumin (dashed line) at 0.1% (w/v) of each protein (A) or 12.5% (w/v) of each protein (B). The proportion of each protein in each fraction was determined by immunological assay. The product of sample concentration and flow rate was 463 times as great in (B) as in (A).

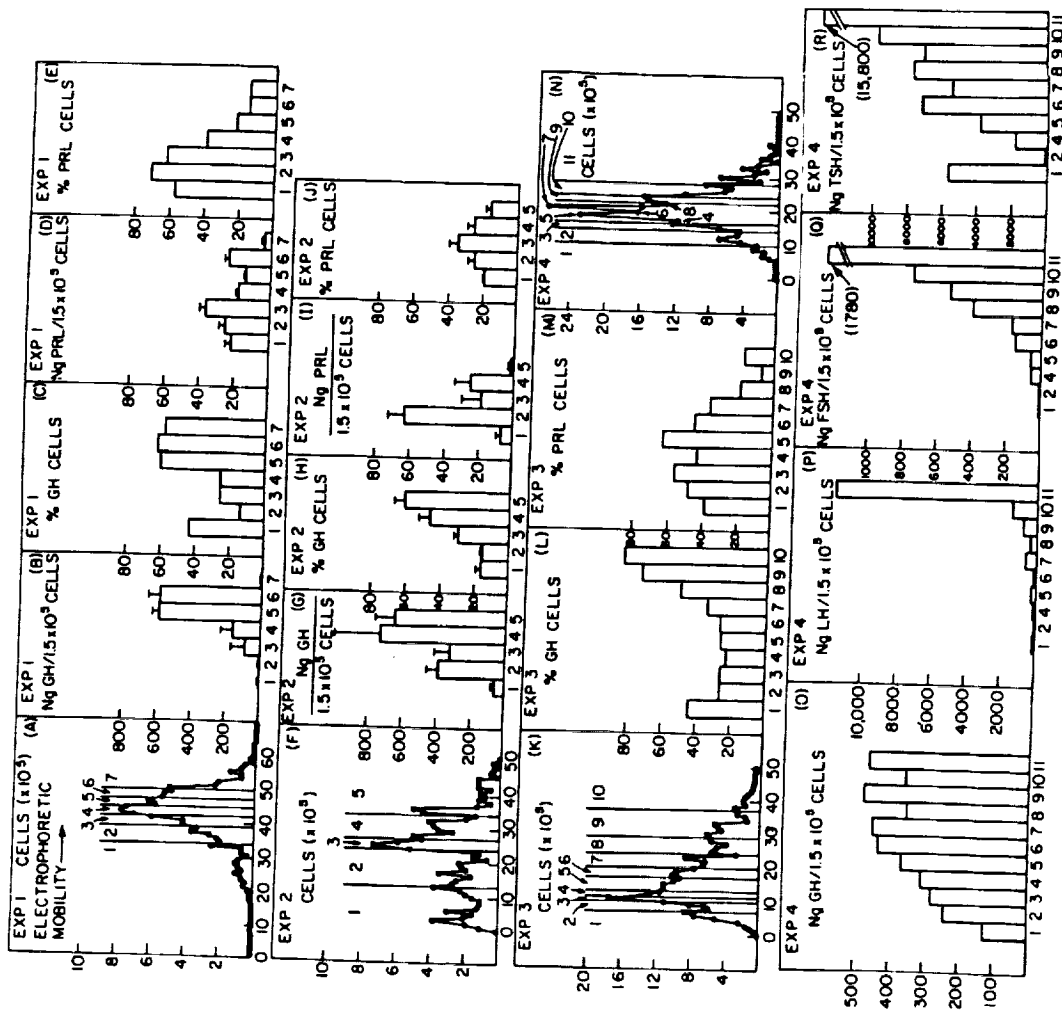
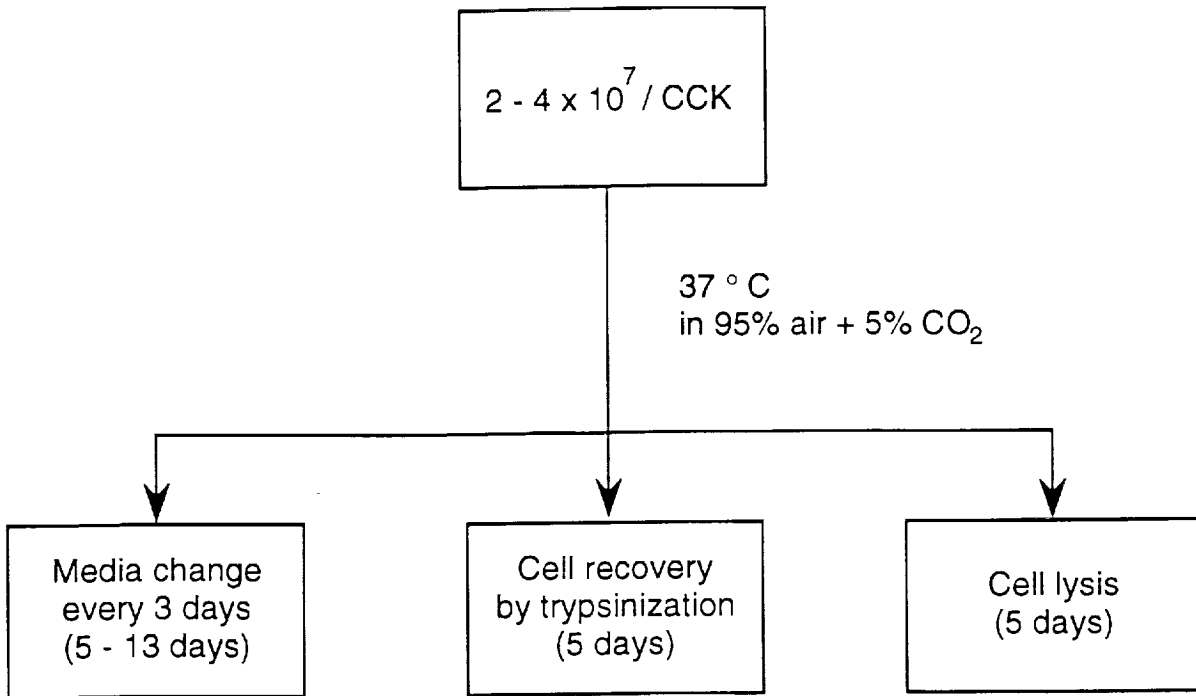


Fig. 4. Results of four rat pituitary cell CFE experiments done under unit gravity (Expts. 1, 3, 4) or microgravity (Expt. 2) conditions. All graphs are distributions among electrophoretic fractions of cells or hormone levels. For details, see text.

## APPENDICES 5-23

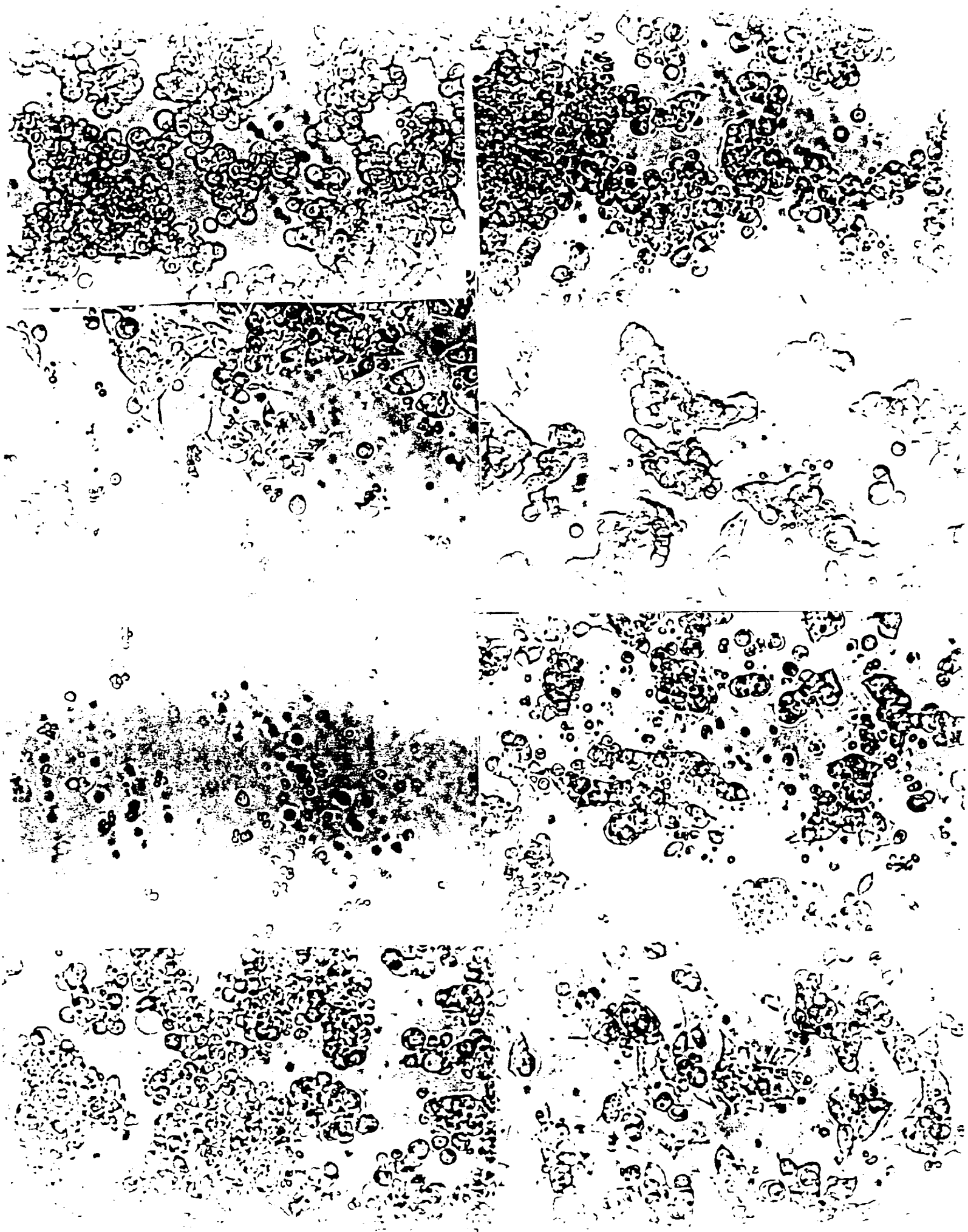


Preflight experiment  
Part 1  
Cell Culture Kit (CCK)\*



- Cell morphology
- GH EIA
- Cell recovery / viability
- Lysis

\* First available 12 / 92

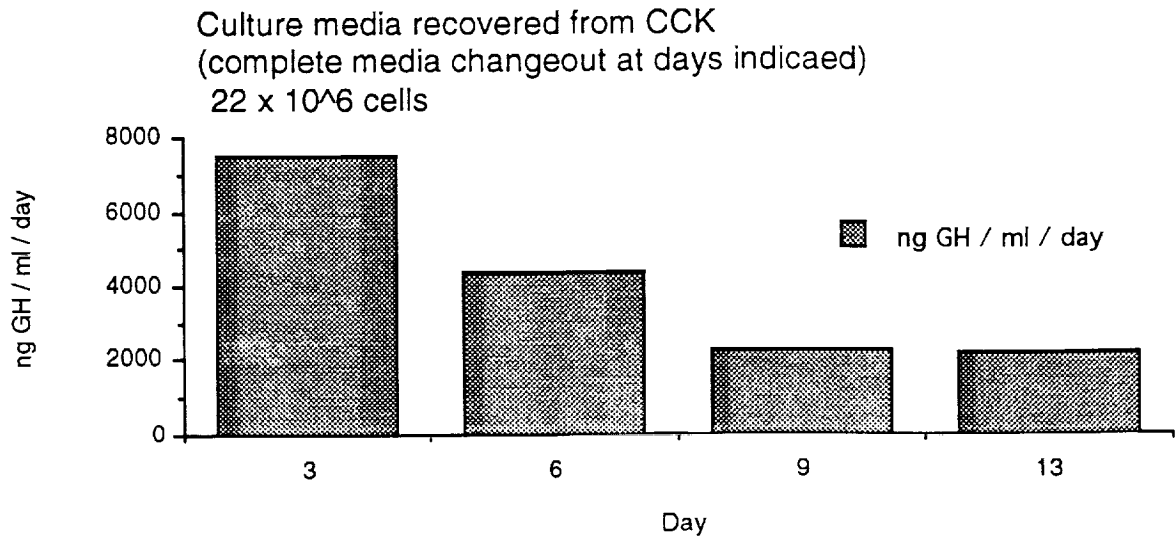


Summary of FFEU cell culture kit (CCK) experiments

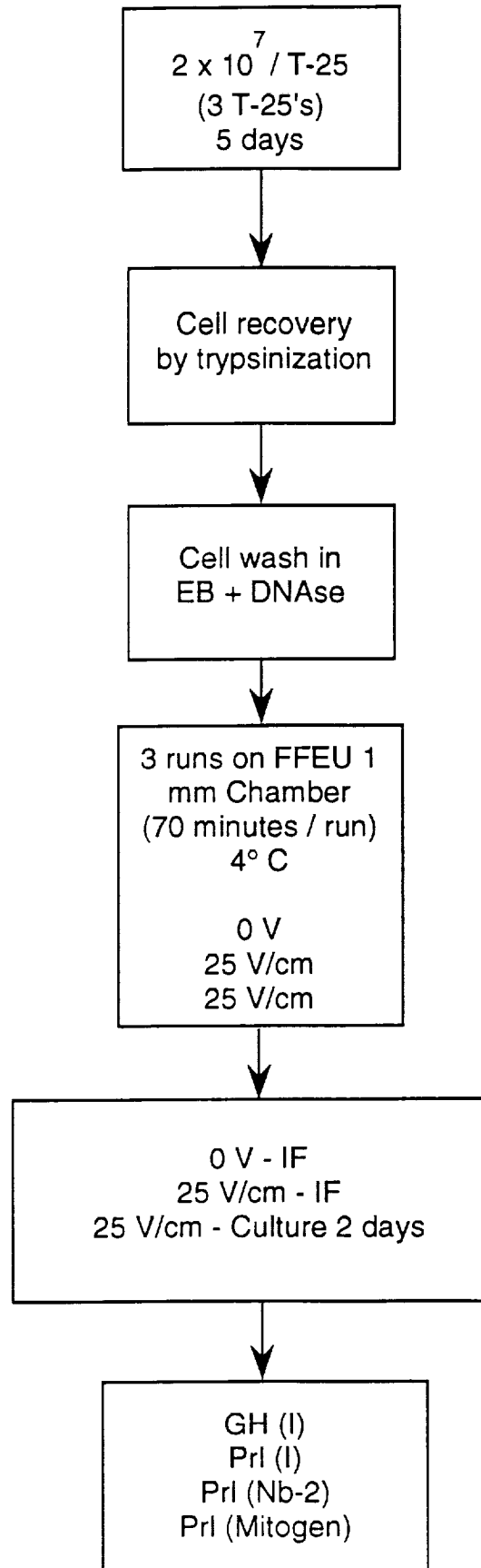
Attempt #	# Cells	Days in culture	% Recovery *	% Viability
1	$22 \times 10^6$	5	0.85	82
2	$22 \times 10^6$	5	3.4	87
3	$22 \times 10^6$	13	12	85
4	$40 \times 10^6$	5	30	99
5	$22 \times 10^6$	5	Lysis complete after 10 minutes**	

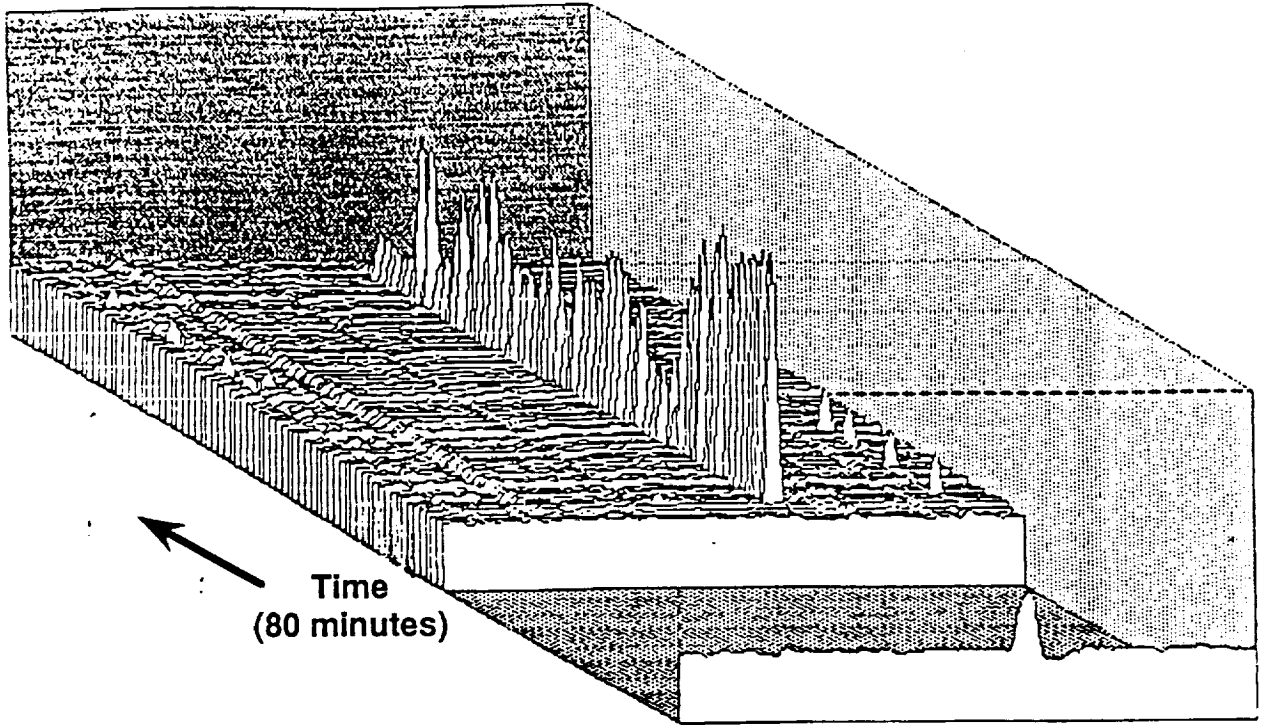
\* ~ 15 minutes for trypsinization

\*\* ~ 10 mls of lysis buffer



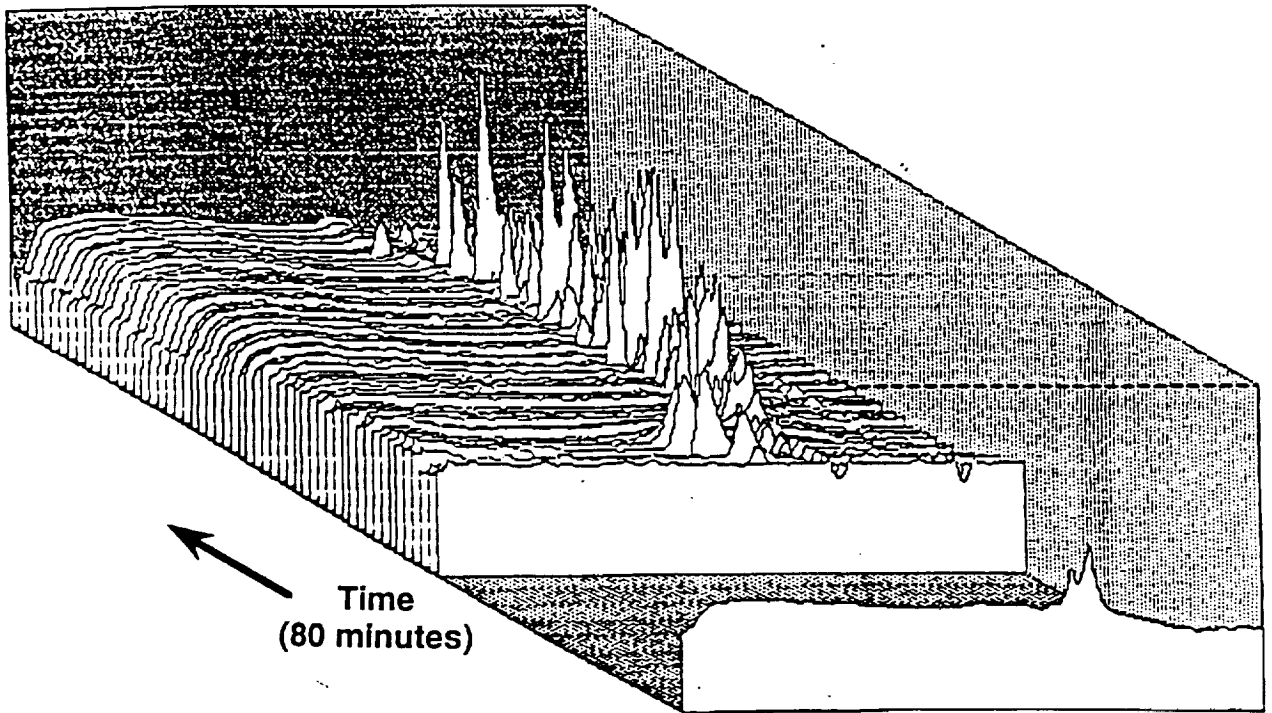
Preflight experiment  
Part 2





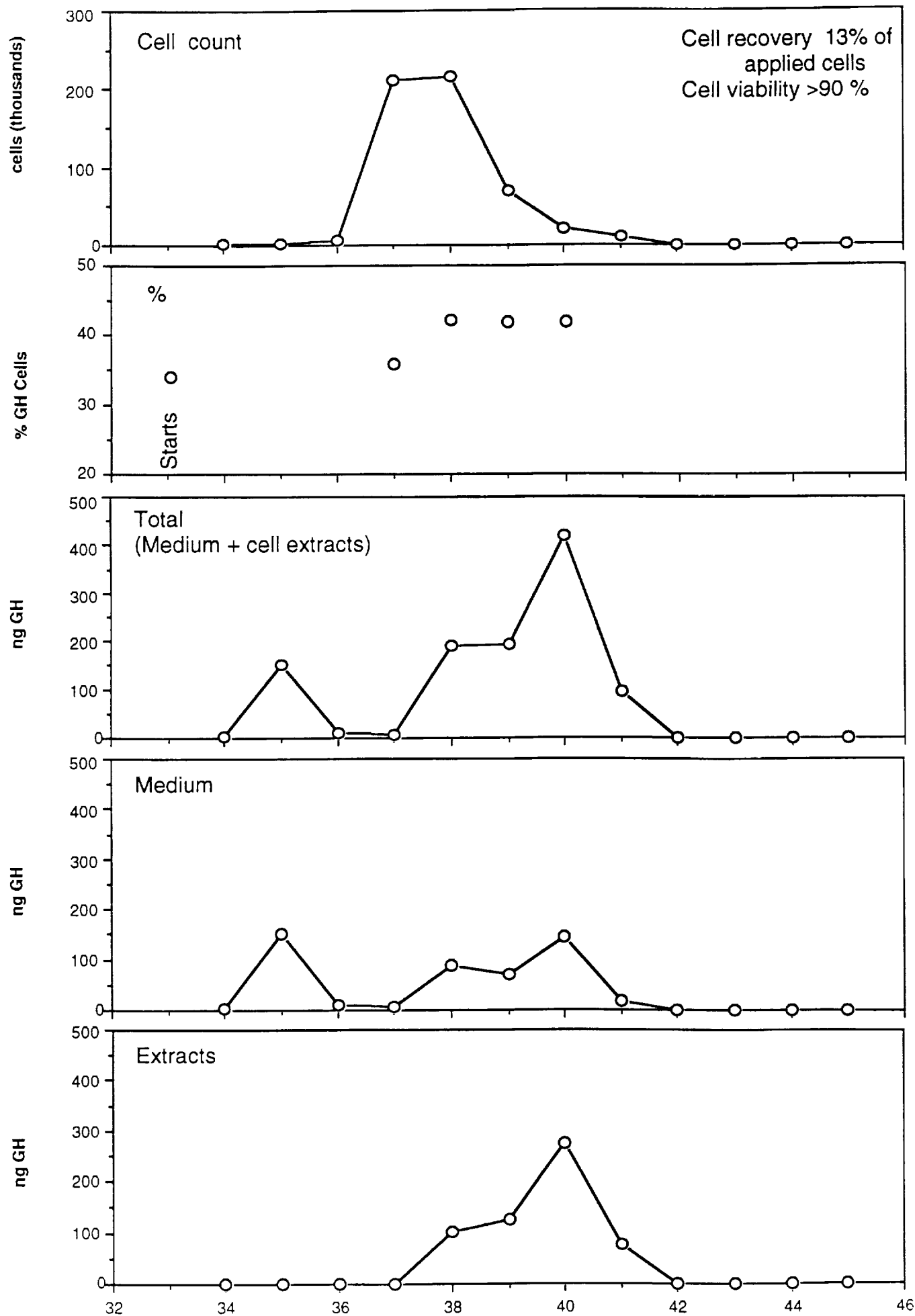
Time  
(80 minutes)

Mobility  
(Zero V/cm)

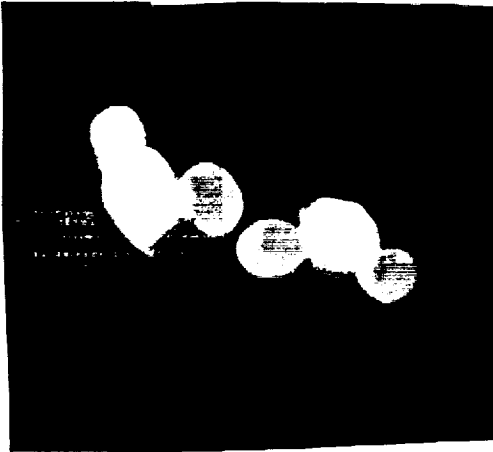


Time  
(80 minutes)

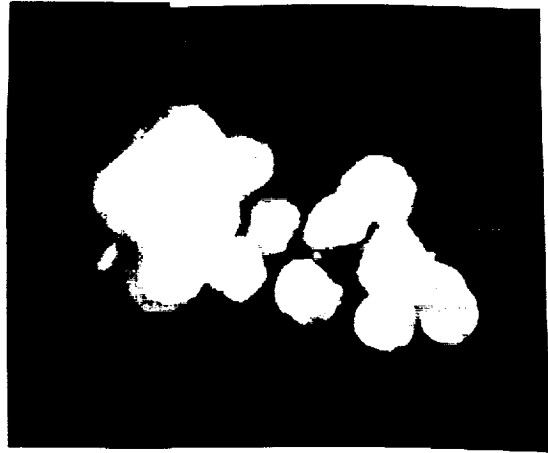
Mobility  
(25 V/cm)



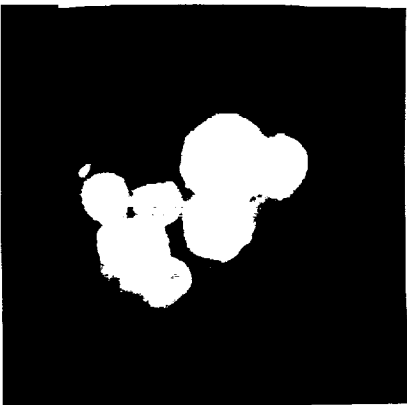
Start



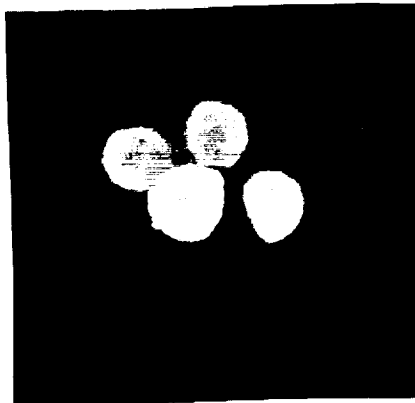
EB Wash



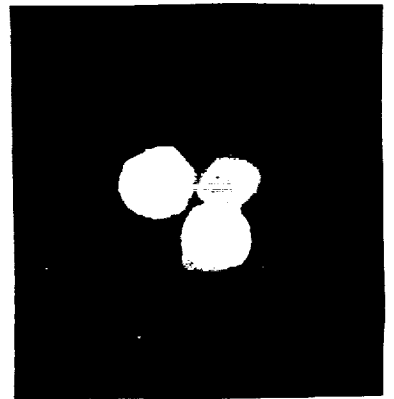
Fraction # 37



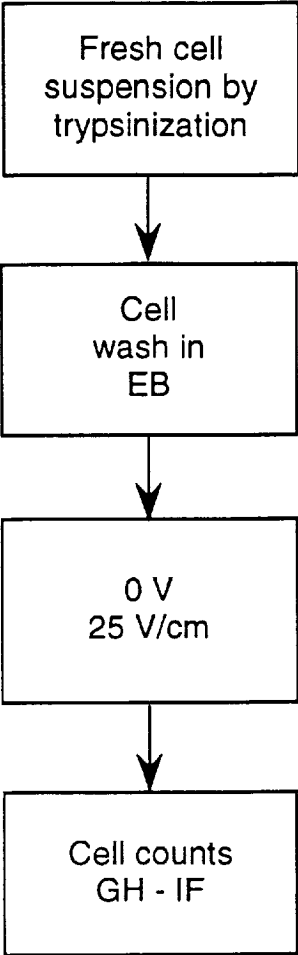
Fraction # 38



Fraction # 39

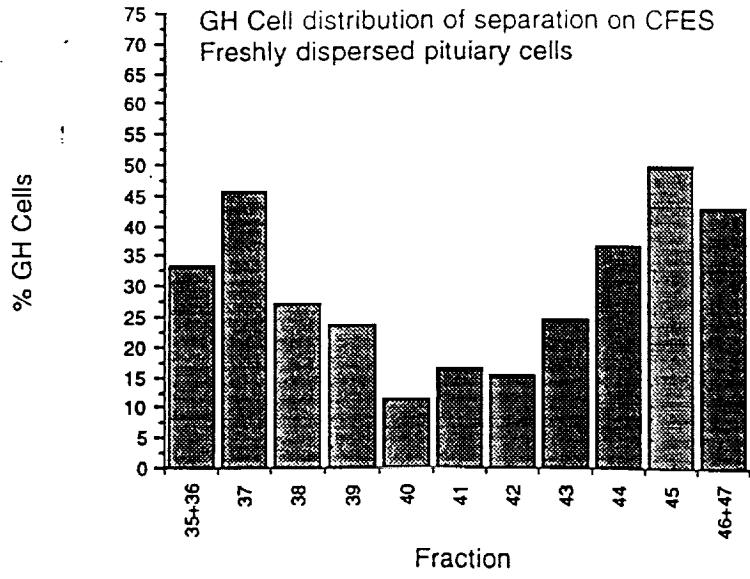


Experimental Series A  
CFES





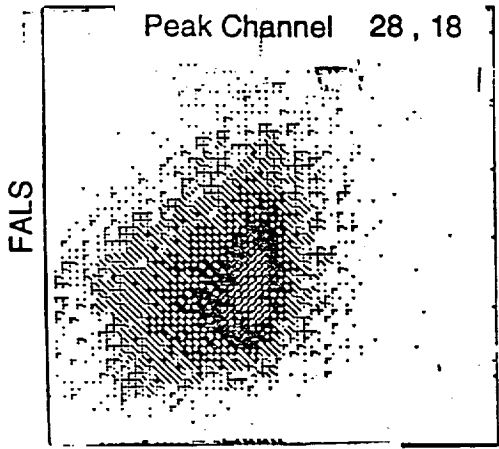
GH Cell distribution of separation on CFES  
Freshly dispersed pituitary cells



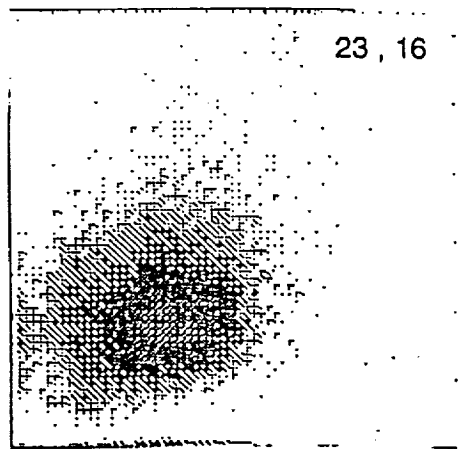
35 / 36

40

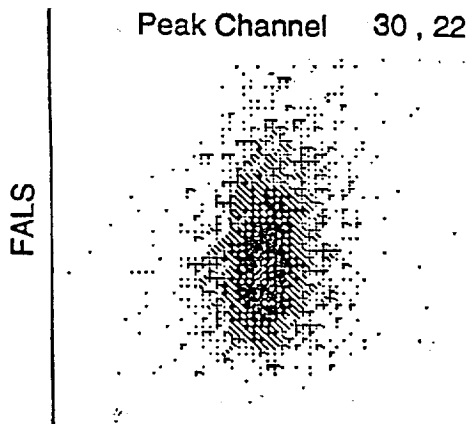
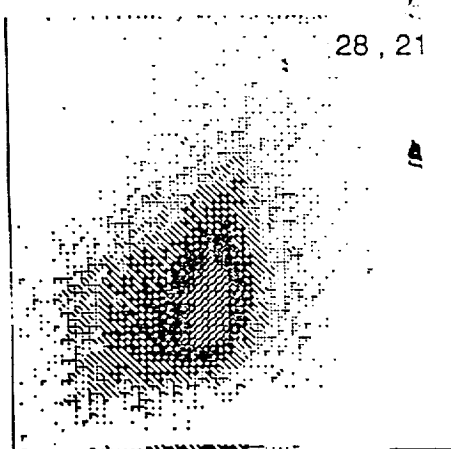
46 / 47



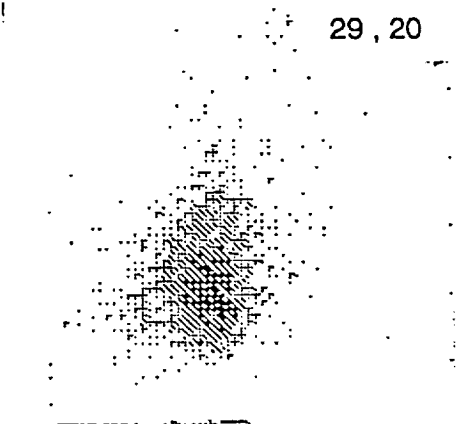
90° Light Scatter



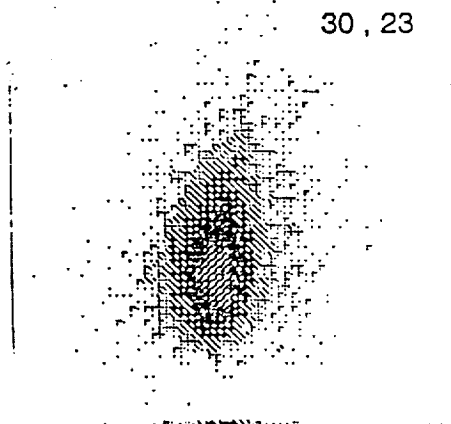
Total cell population



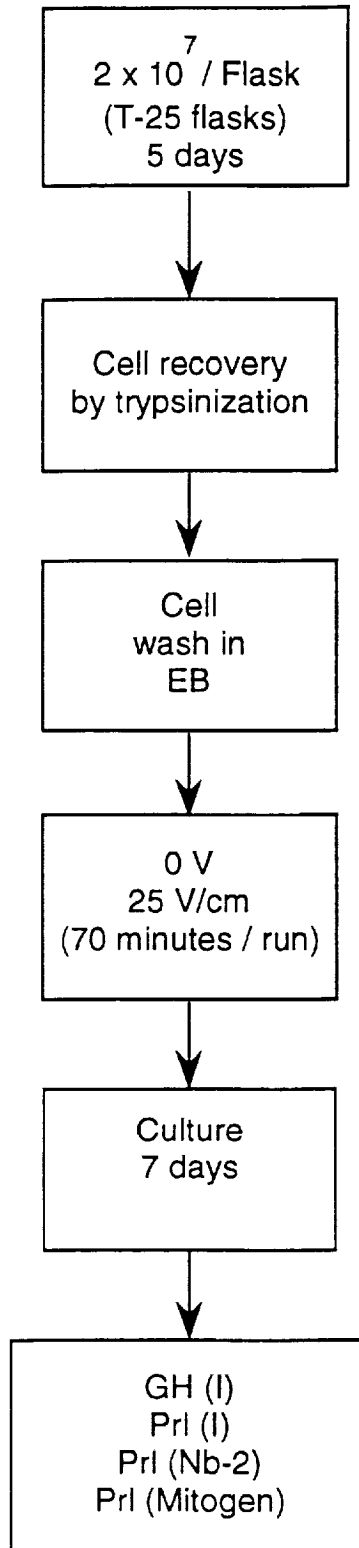
90° Light Scatter



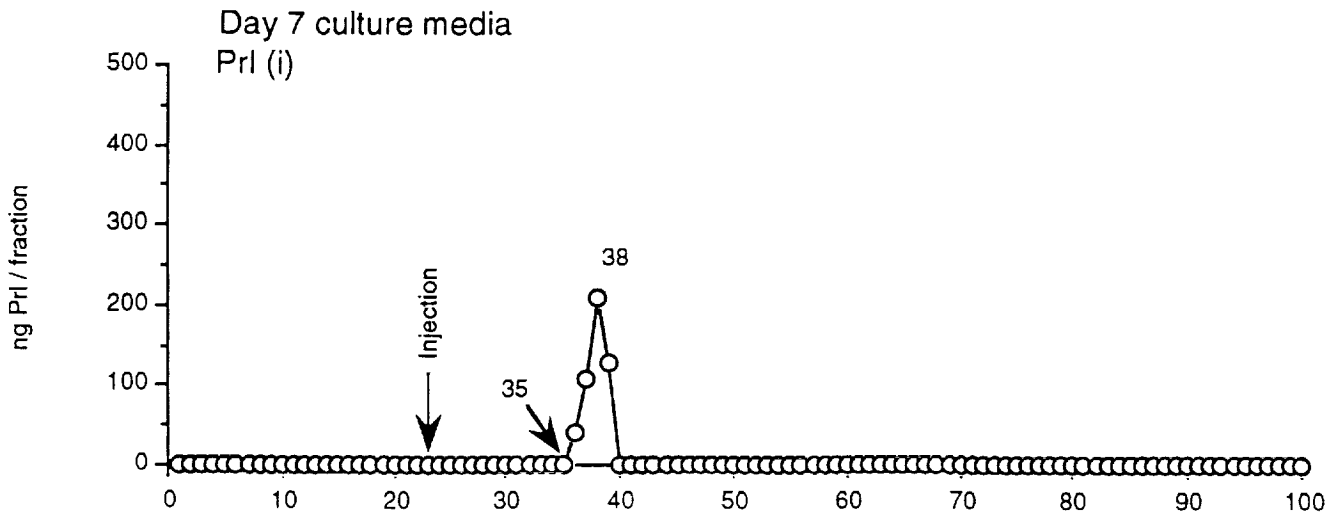
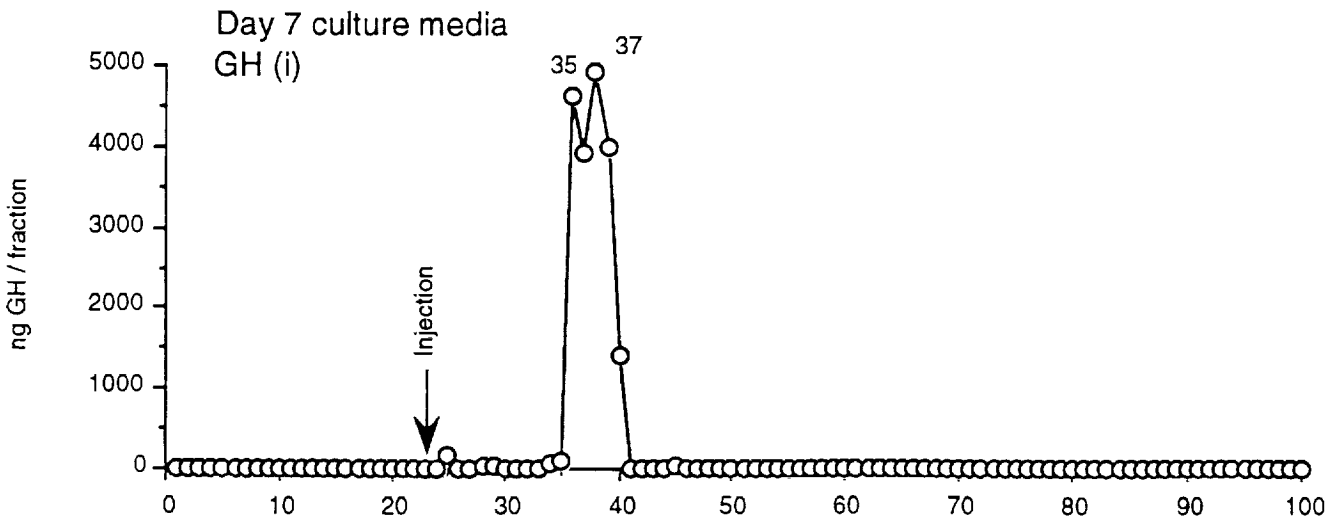
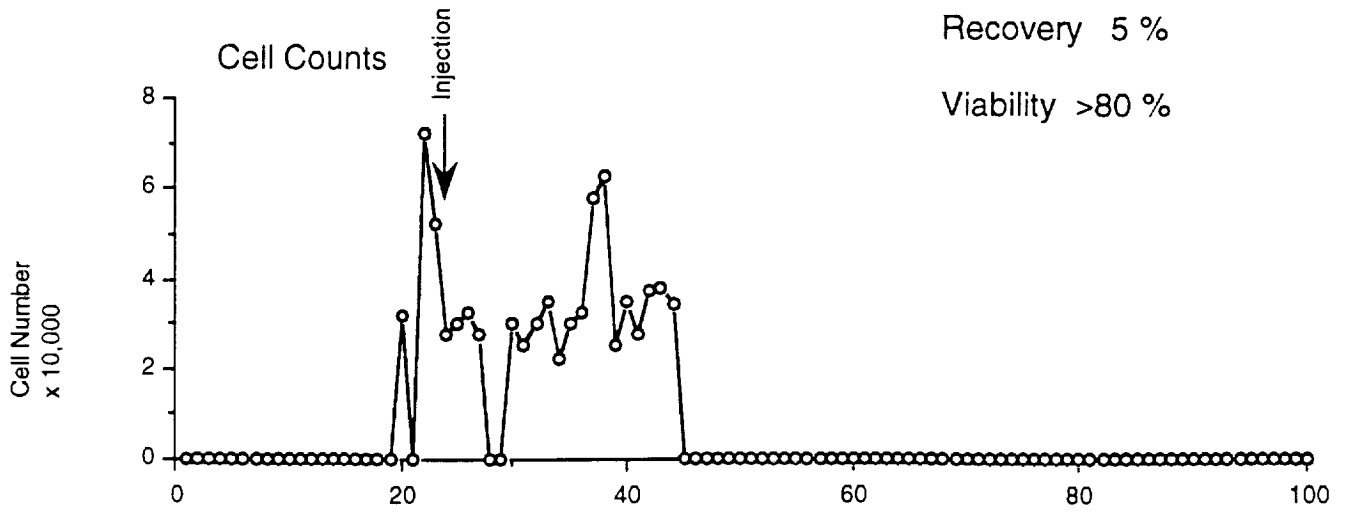
GH positive cells only



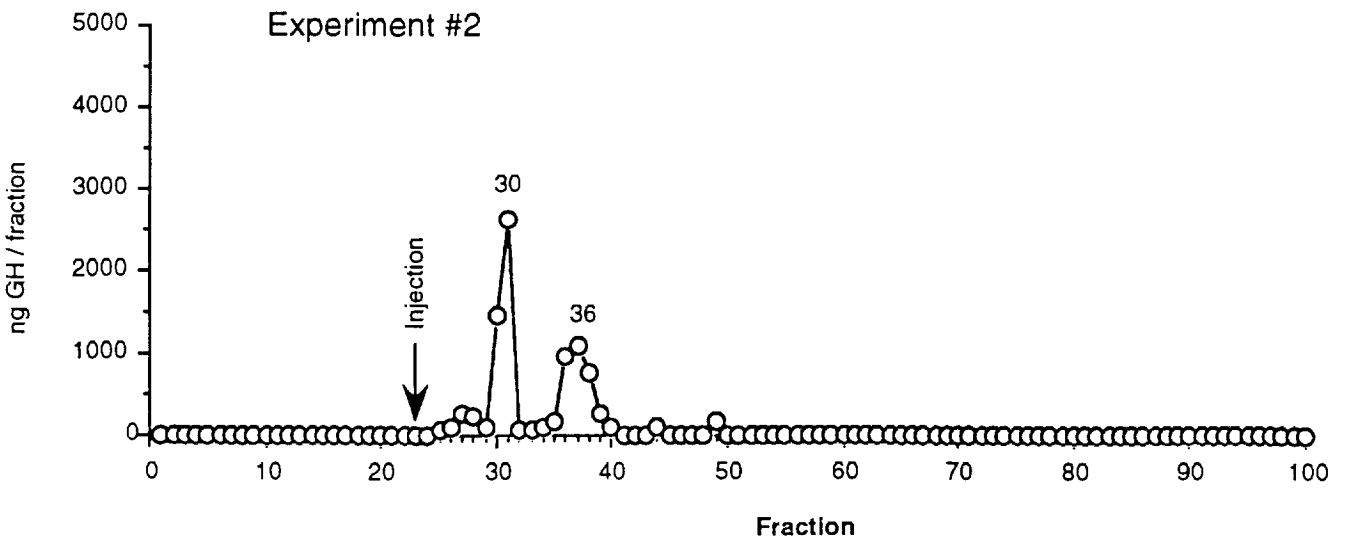
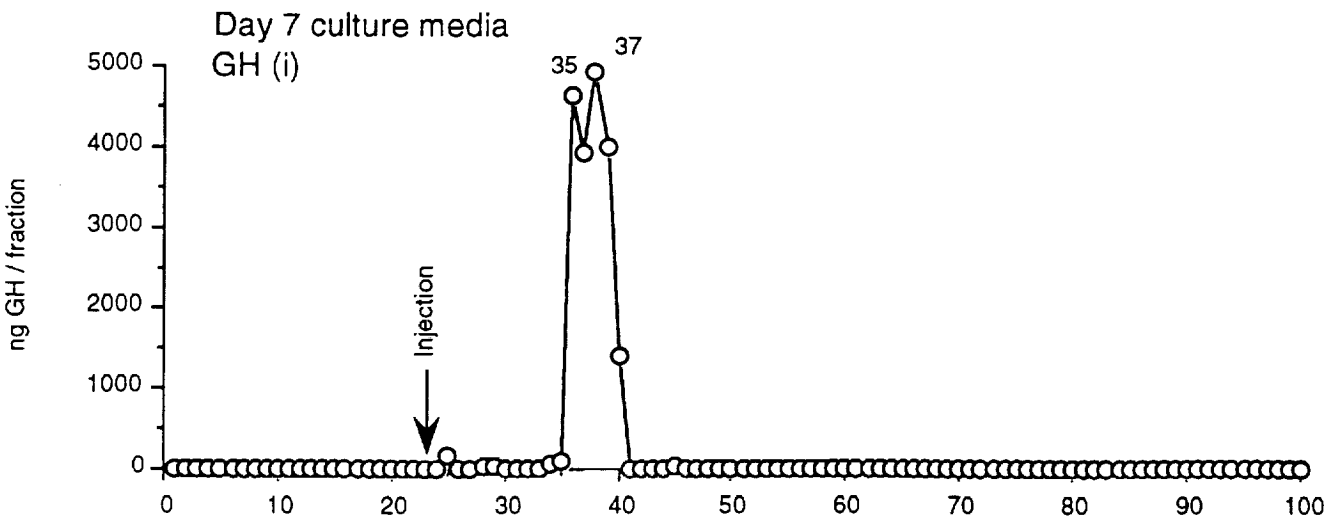
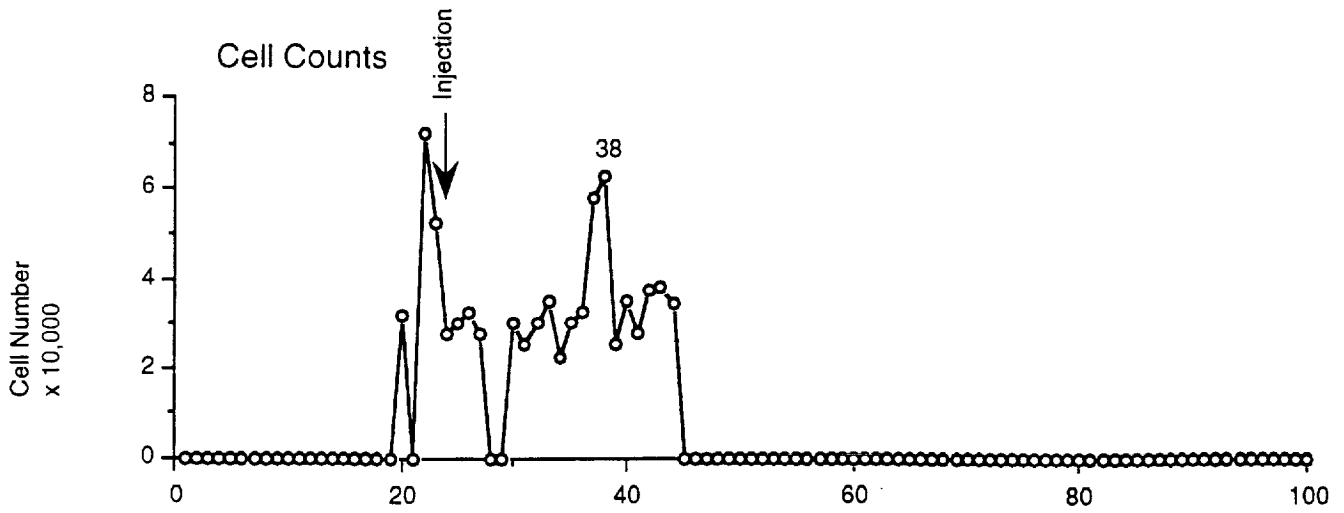
Experimental Series B



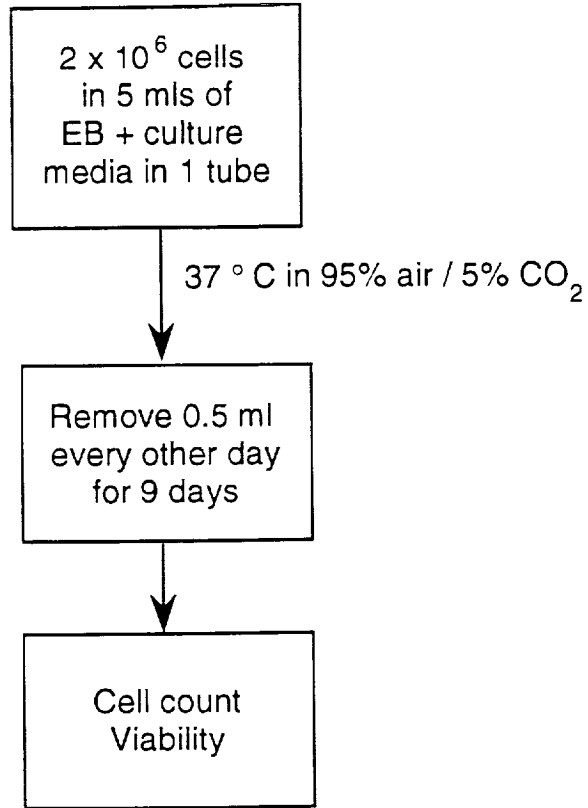
Experimental Series B



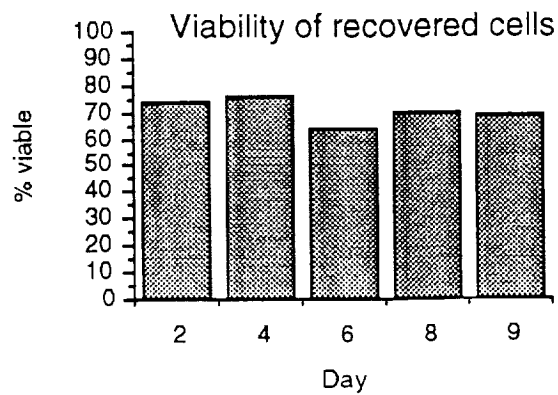
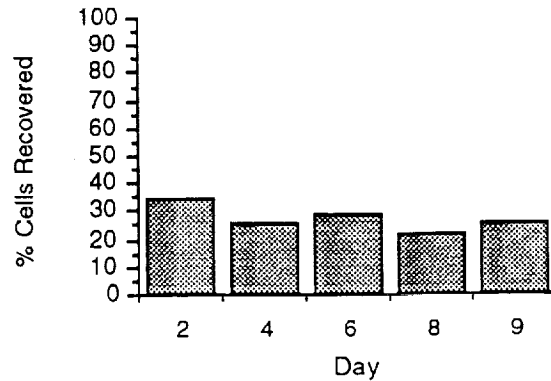
GH content of media after separating cells on CFES  
In culture for 7 days



# Cell viability in FFEU collection tube

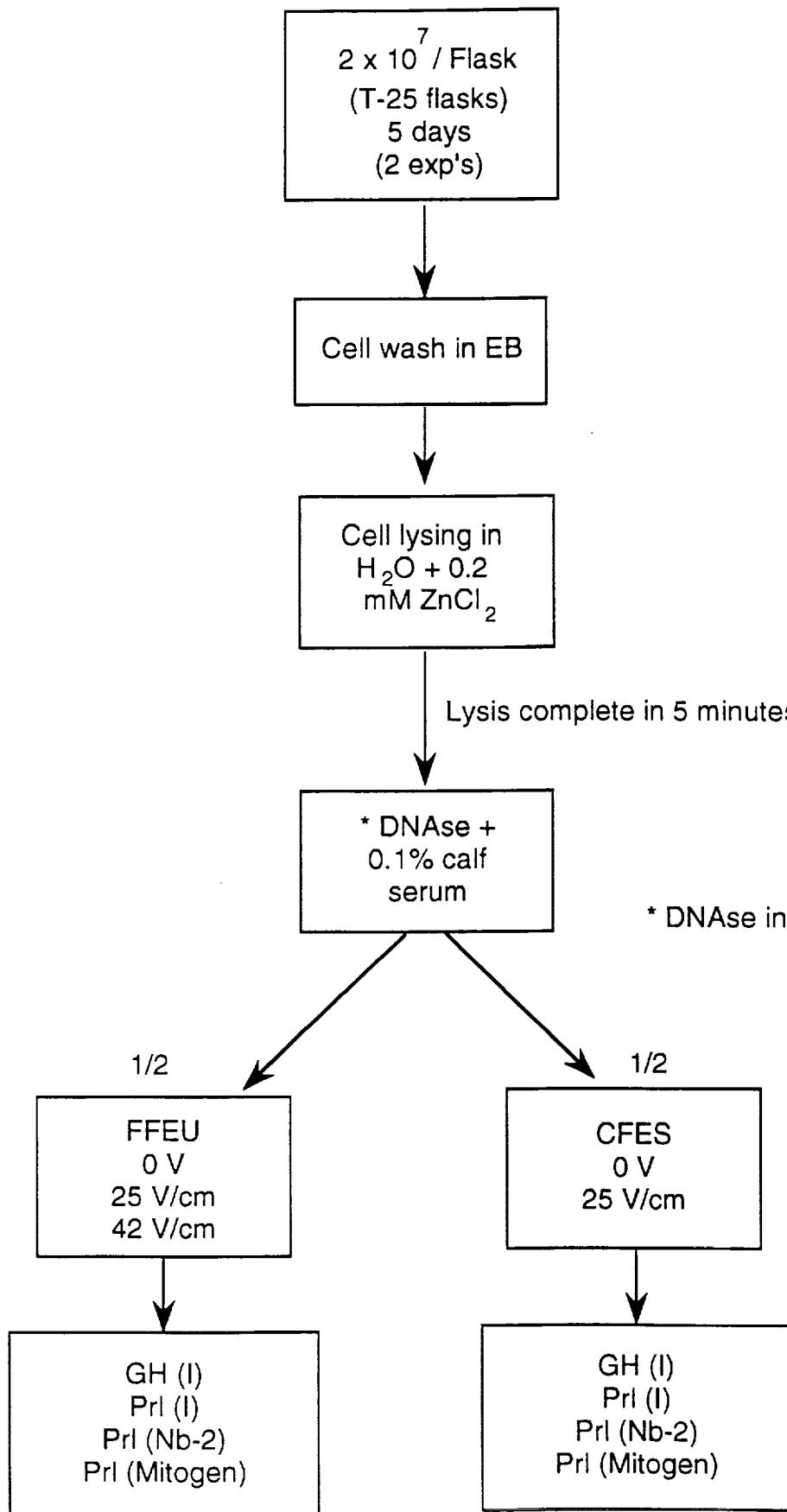


## Cells recovered as a percent of cells seeded

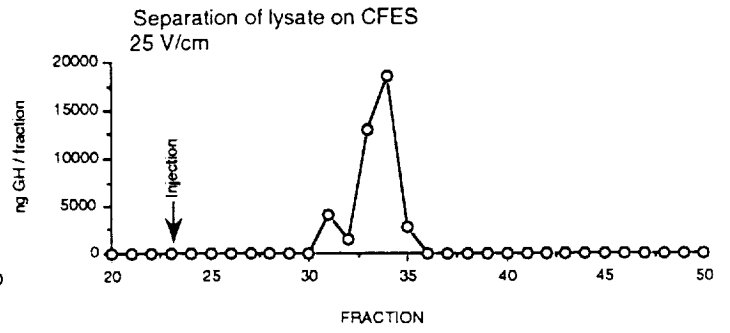
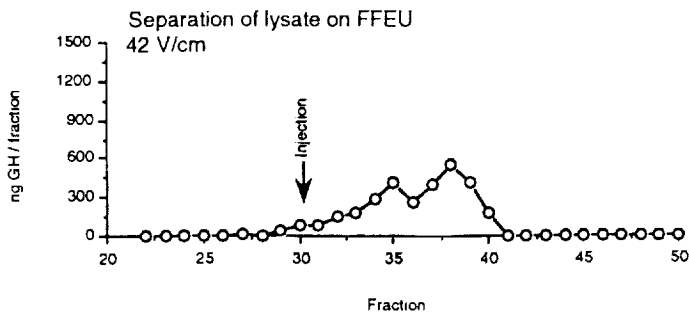
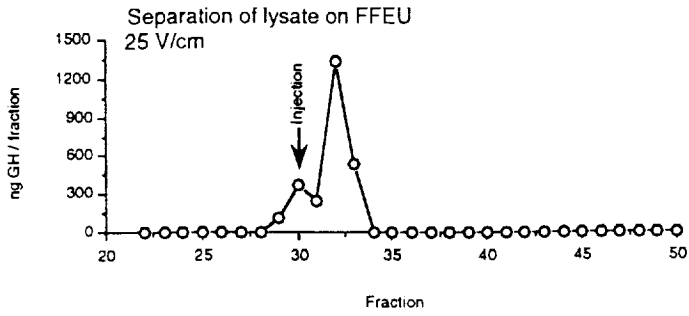
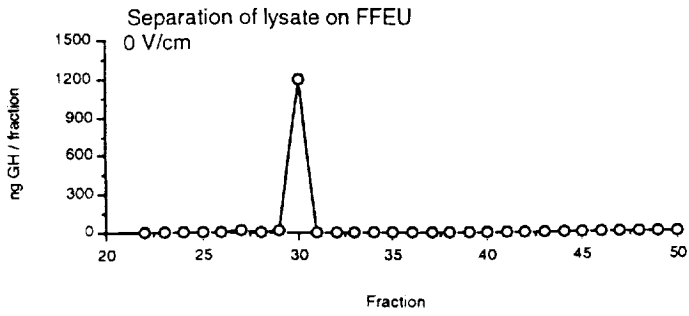


- Conclusions:
- 1) Cells may "stick" to membrane
  - 2) awaiting additional collection tubes from NASDA
  - 3) Possibilities to increase cell recovery
    - washing
    - trypsinization (TBD)

Preflight experiments for Part 3



\* DNase in lysing solution? (TBD)

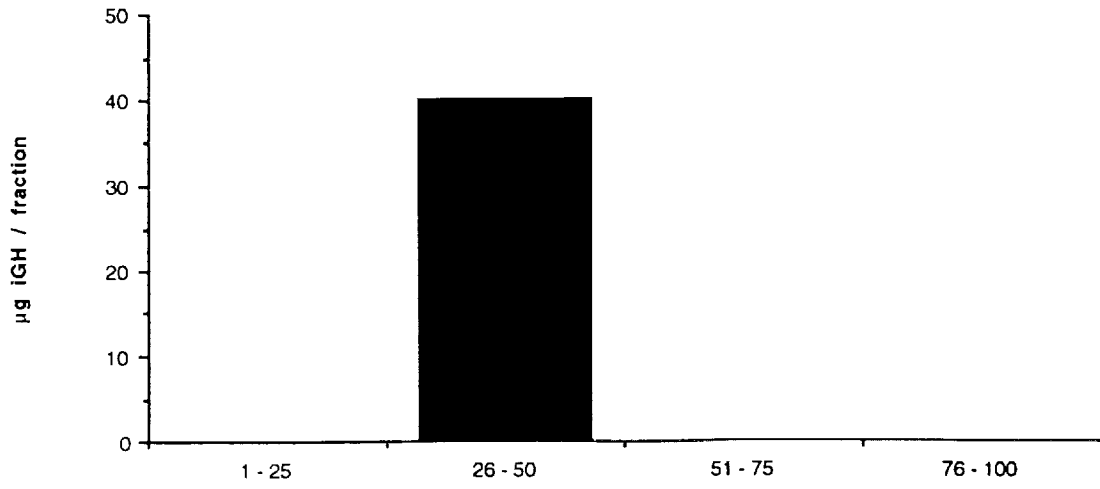


	% Processed	% GH Recovered (Total)
FFEU	13	150
CFES	69	180

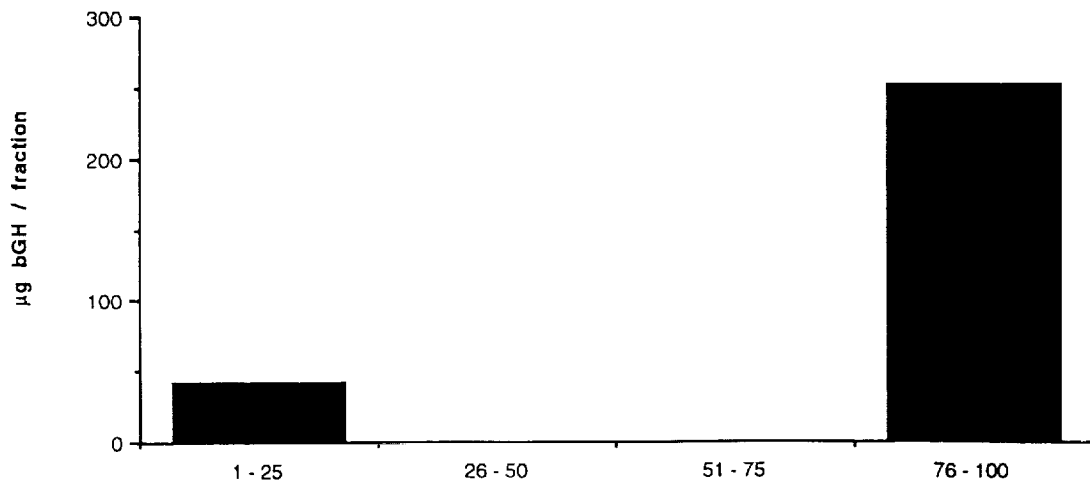
FFEU: 2.7 mls → 1.35 ml → 0.177 ml / run @ 0.15 ml / hr

CFES 5.4 mls → 3.75 ml / run @ 2.5 ml / hr

Lysate separated on CFES

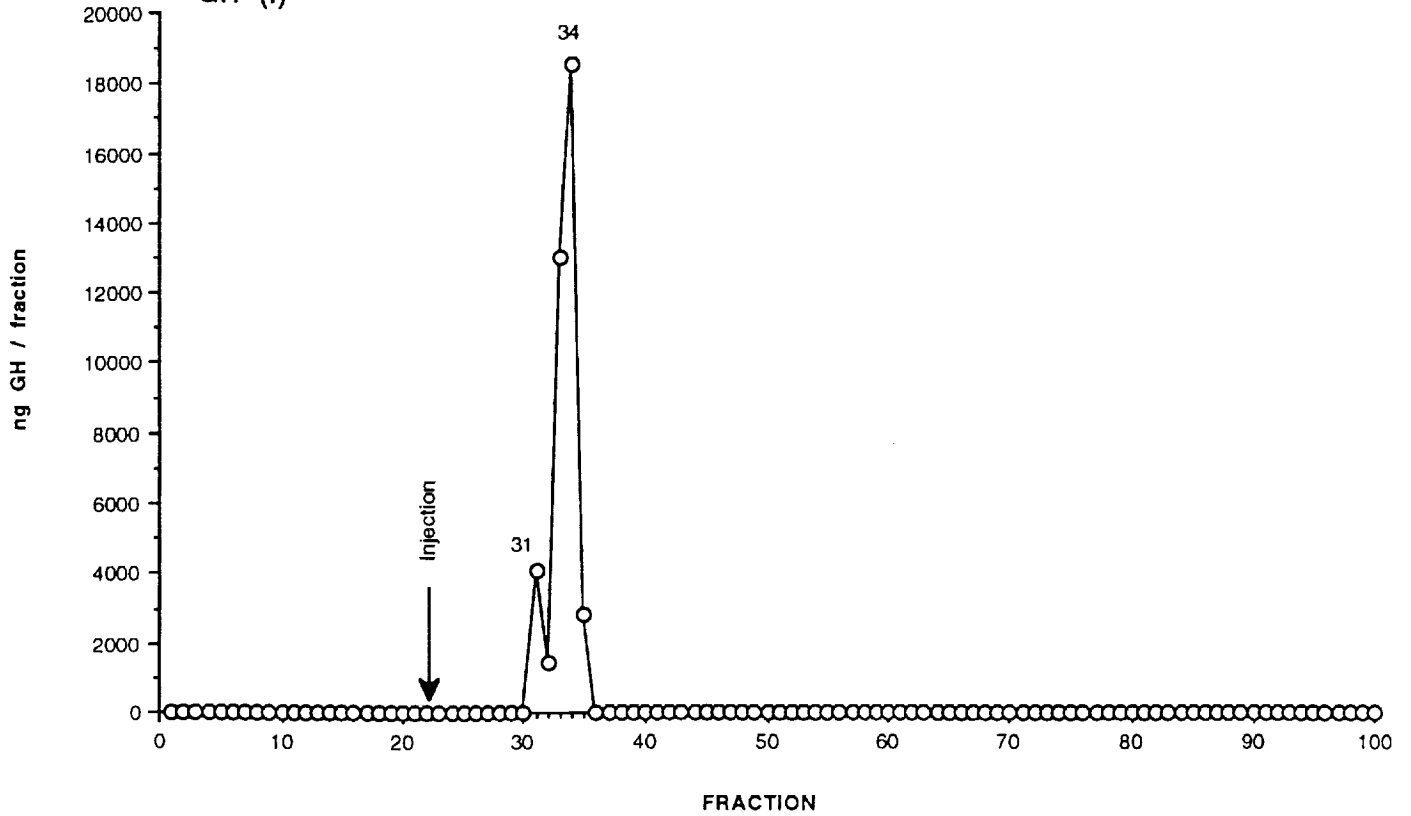


Lysate separated on CFES

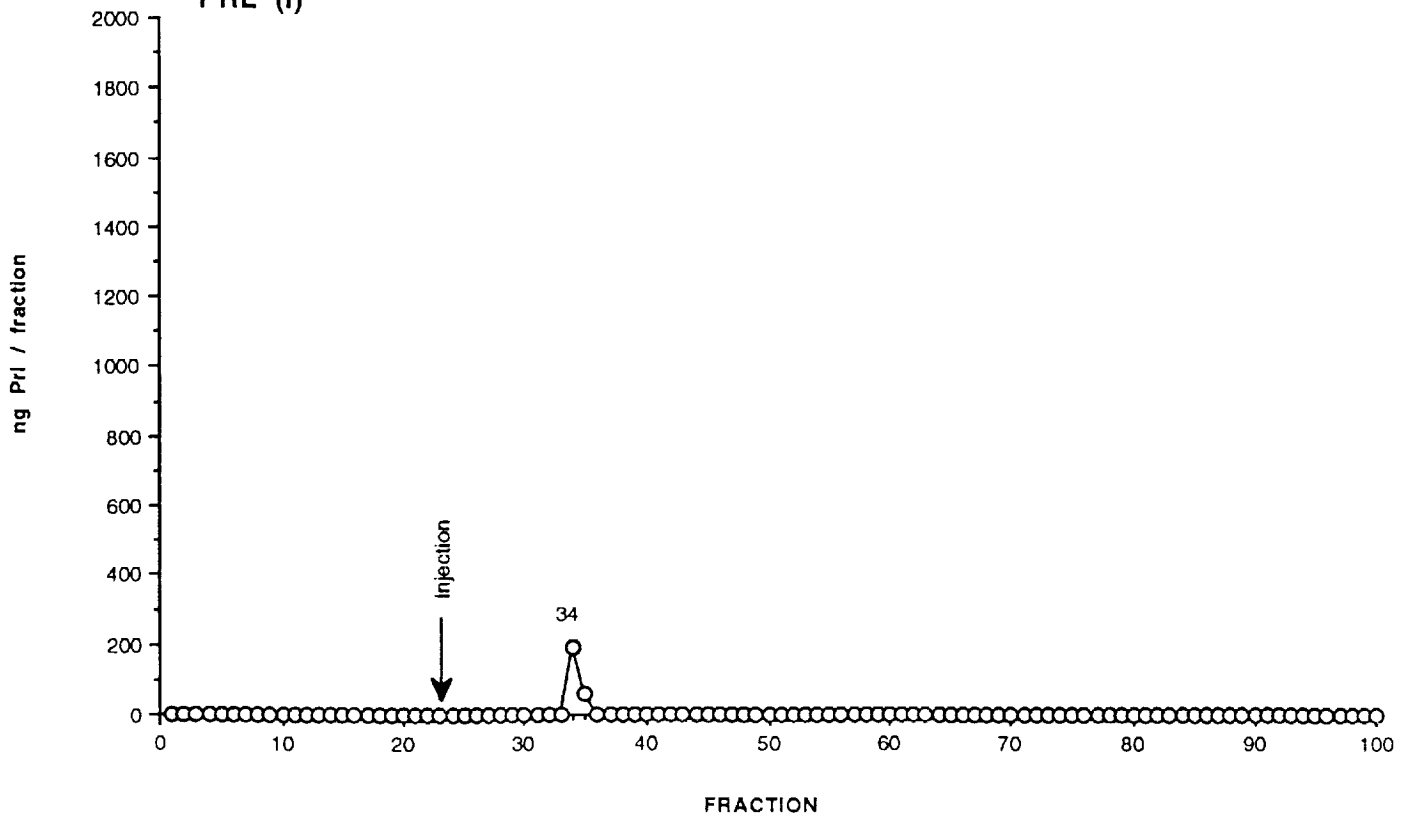




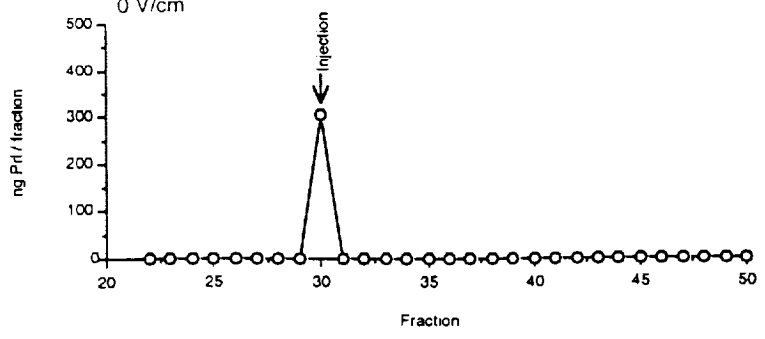
CFES Separation of pituitary cell lysate  
25 volts / cm  
GH (I)



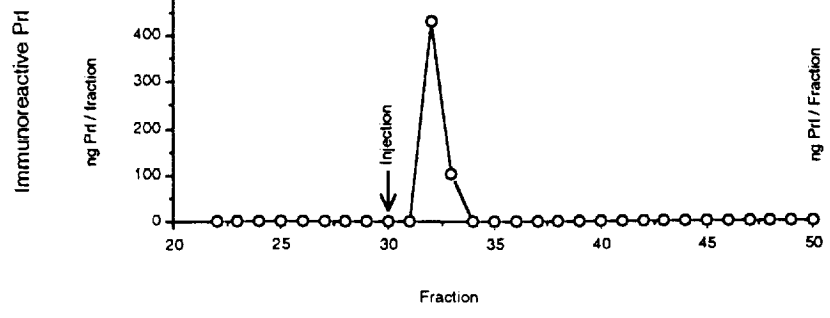
CFES Separation of pituitary cell lysate  
25 volts / cm  
PRL (I)



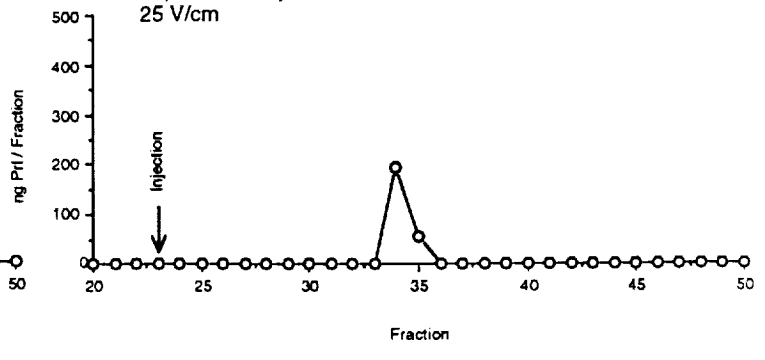
Separation of lysate on FFEU



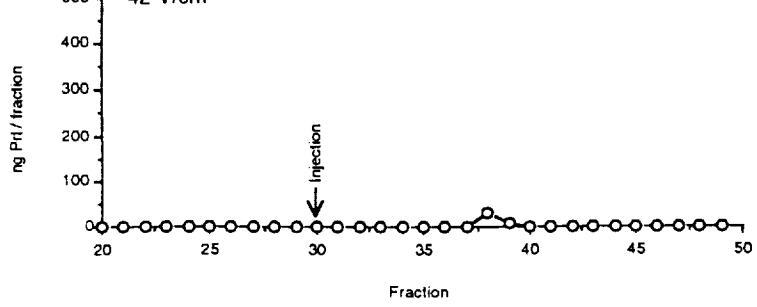
Separation of lysate on FFEU  
25 V/cm



Separation of lysate on CFES  
25 V/cm



Separation of lysate on FFEU  
42 V/cm

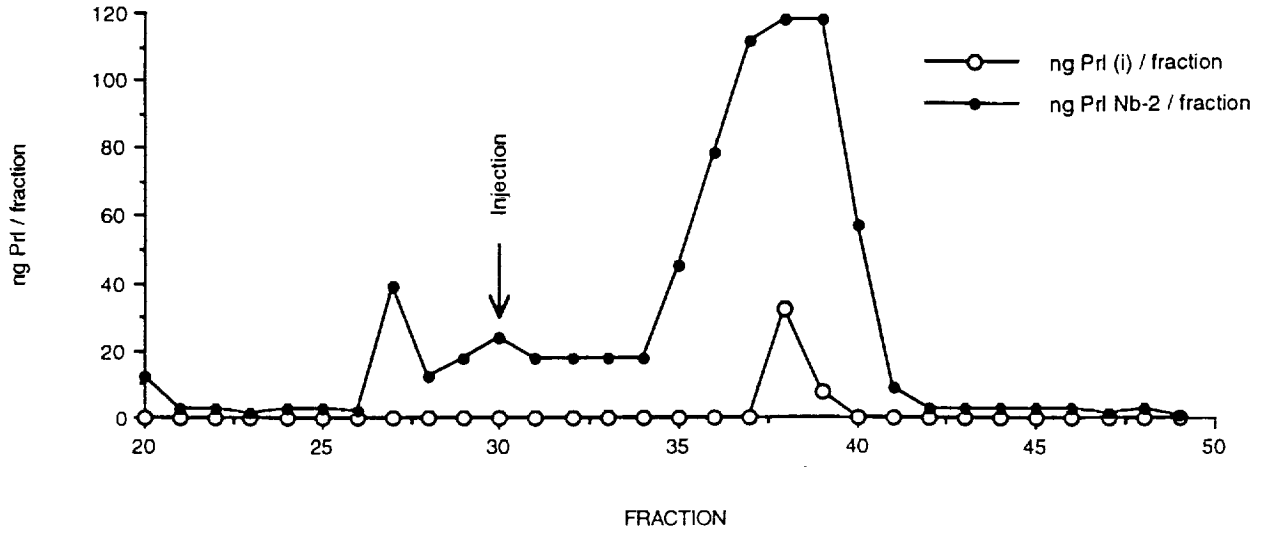


	% Processed	% Prt Recovered (Total)
FFEU	13	7.8
CFES	69	1.8

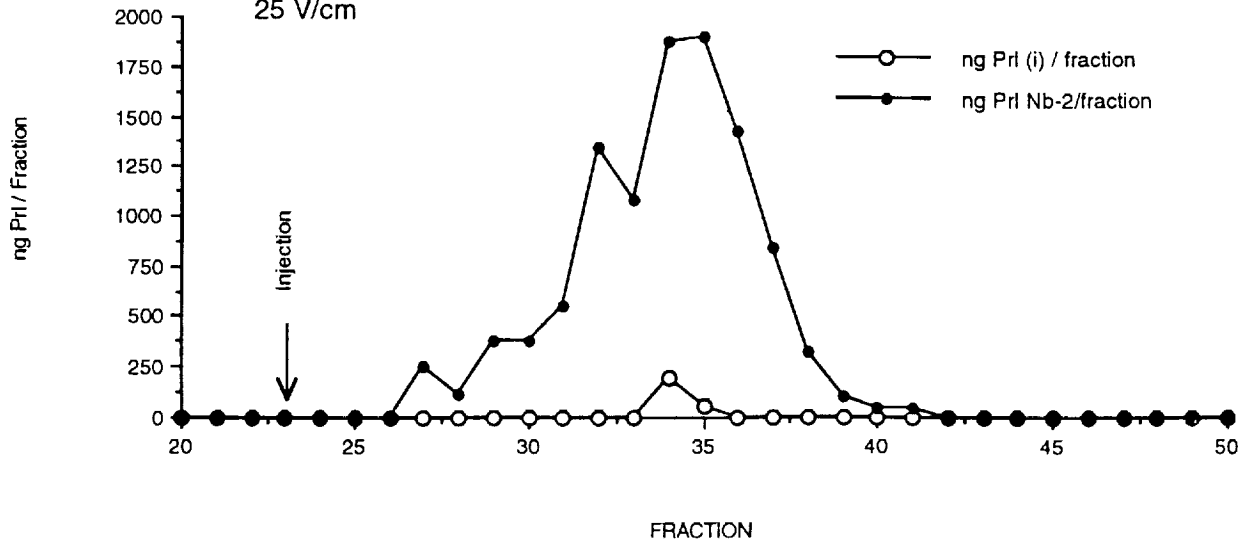
FFEU: 2.7 mls → 1.35 ml → 0.177 ml / run @ 0.15 ml / hr

CFES 5.4 mls → 3.75 ml / run @ 2.5 ml / hr

Separation of lysate on FFEU  
42 V/cm



Separation of lysate on CFES  
25 V/cm



## PUBLICATIONS USING CFE

### REVIEWED

Plank, L., W.C. Hymer, E. Kunze, G. Marks, J. Lanham and P. Todd. 1983. A study of cell electrophoresis as a means of purifying growth hormone secreting cells. J. Biochem. and Biophys. Methods 8:275-289.

Zarkower, D., L. Planck, E. Kunze, P. Todd and W.C. Hymer. 1984. Alteration by prolactin of surface charge and membrane fluidity of rat 13762 mammary ascites tumor cells. Cell Biophysics 6:53-66.

Morrison, D.R., G.H. Barlow, C. Cleveland, M. Farrington, R. Grindeland, J.M. Hatfield, W.C. Hymer, J.W. Lanham, M.L. Lewis, D.S. Nachtwey, P. Todd and W. Wilfinger. 1984. Electrophoretic separation of kidney and pituitary cells on STS-8. Advances Space Research 4:67-76.

Hymer, W.C., G.H. Barlow, S.J. Blaisdell, C. Cleveland, M. Farrington, M. Feldmeier, R. Grindeland, J.M. Hatfield, J.W. Lanham, M.L. Lewis, D.R. Morrison, B.J. Olack, D.W. Richman, J. Rose, D.W. Scharp, R.S. Snyder, C.A. Swanson, P. Todd and W. Wilfinger. 1987. Continuous flow electrophoresis separation of proteins and cells from mammalian tissues. Cell Biophysics 10:61-85.

Hayes, D., C. Exton, T. Salada, K. Shellenberger, J. Waddle and W.C. Hymer. 1990. Separation of rat pituitary secretory granules by continuous flow electrophoresis. Electrophoresis 11:976-978.

### CHAPTERS/PROCEEDING

Boltz, R.C. Jr., P. Todd, R.H. Hammerstedt, W.C. Hymer, C.J. Thompson and J. Docherty. 1977. Initial studies on the separation of cells by density gradient isoelectric focusing, in Cell Separation Methods, ed. H. Bleomendal, Elsevier/North Holland Biomedical Press, New York, pp. 143-155.

Todd, P., W.C. Hymer, L.D. Plank, G.M. Marks, M. Hershey, V. Giranda, M.E. Kunze and R.R. Mehrishi. 1981. Separation of functioning mammalian cells in density-gradient electrophoresis, in Electrophoresis '81, eds. R.C. Allen and P. Arnaud, W. DeGreuter Press, New York, pp. 871-882.

Hymer, W.C. and J.M. Hatfield. 1984. Separation of cells from the rat anterior pituitary gland, in Cell Separation: Methods and Selected Applications, ed. T.G. Pretlow and T. Pretlow. Academic Press, Vol. 3, pp. 163-194.

Hymer, W.C., R. Grindeland, J.W. Lanham and D. Morrison. 1985. Continuous flow electrophoresis (CFE): Applications to growth hormone (GH) research and development. Proceedings, Pharm Tech Conference, pp. 13-19.

Hymer, W.C., R. Grindeland, C. Hayes, J. Lanham and D. Morrison. 1987. Life sciences, biotechnology and microgravity, in Advances in the Astronautical Sciences, Vol. 61. Edited by G.W. Morgenthaler, G.L. May, W.K. Tobiska and J.N. Koster. American Astronautical Society by Univelt, Inc., pp. 1333-1345.

Todd, P., W.C. Hymer, D.R. Morrison, C.L. Goolsby, J.M. Hatfield, M.E. Kunze and K. Motter. 1987. Cell bioprocessing in space: applications of analytical cytology. Ninth Annual Meeting IUPS Commission on Gravitational Physiology, Czechoslovakia, September 28 - October 1987.

Hymer, W.C., R. Grindeland, C. Hayes, J.W. Lanham, P. Todd and D. Morrison. 1988. Heterogeneity in the growth hormone pituitary gland "system" of rats and humans: Implications to microgravity based research. NASA Technical Memorandum 4069. Vol. 1.

### Pituitary

- Relative to cells in standard culture medium (SMEM), recovery is consistently superior in D2/A1 electrophoresis buffer vs. that in Goodwin, Hanning or STS-8 buffers (Fig. 1). Storage temperature has little effect on cell recovery. Figure 1 represents an average of three experiments.
- Cell viability, relative to that in SMEM, is again best in D2/A1 buffer (Figure 2).
- Storage of cells in D2/A1 buffer for 72 hours at 4°C is the best combination of conditions tried to date.
- Application of rat pituitary homogenates (prepared in 0.25M sucrose + 2 mM 2 nCL<sub>2</sub>) to the McDonnell Douglas continuous flow electrophoresis (CFE) unit yields several peaks of particles containing rat growth hormone (GH). These are incompletely separated and show anodal migration (Figure 3; zero tube is #23, 24). MES-gly buffer. Results are repeatable.
- Application of a 40,000 x g pellet fraction (known to be enriched in secretion granules) to the McDonnell Douglas CFE shows separation of a major GH-containing partial peak (~ Fr. 40-55) and a minor one just anodal to the injection part.
- These electrophoresis results show that granules can be separated by CFE.

### Splenocytes

- Four electrophoresis buffers were tested for effects on splenocyte viability and recovery at two temperatures 4°C and 22°C and over a

period of four days. Buffers were compared to phosphate buffered saline (PBS) a standard, isotonic cell culture buffer.

- Cell viability was very poor after 24 hours at 22°C in all electrophoresis buffers (Figure 5). Therefore no further studies were done at 22°C. At 4°C the best buffer for recovering viable cells was D2/A1 (Figure 6).
- Mitogen assays were performed on cells after 24 hours in Goodwin's buffer which had high viability in spite of low recovery. Cells responded to Concanavalin A. The other buffers are yet to be tested.

Figure 1

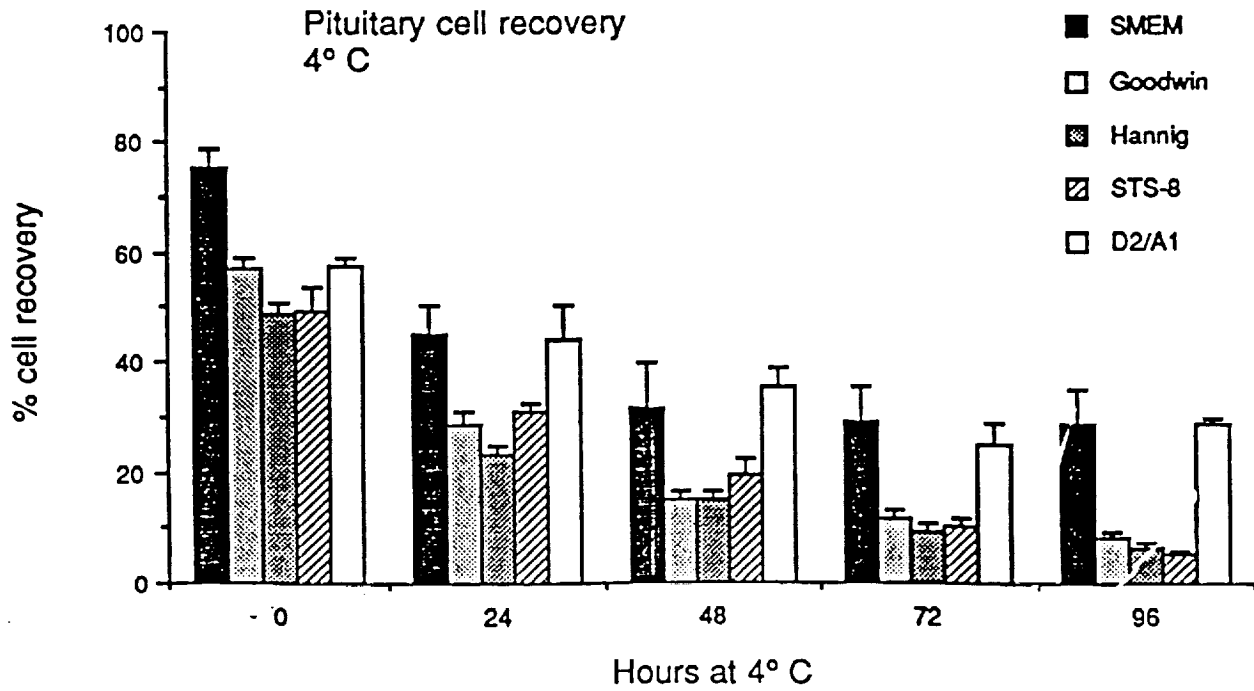
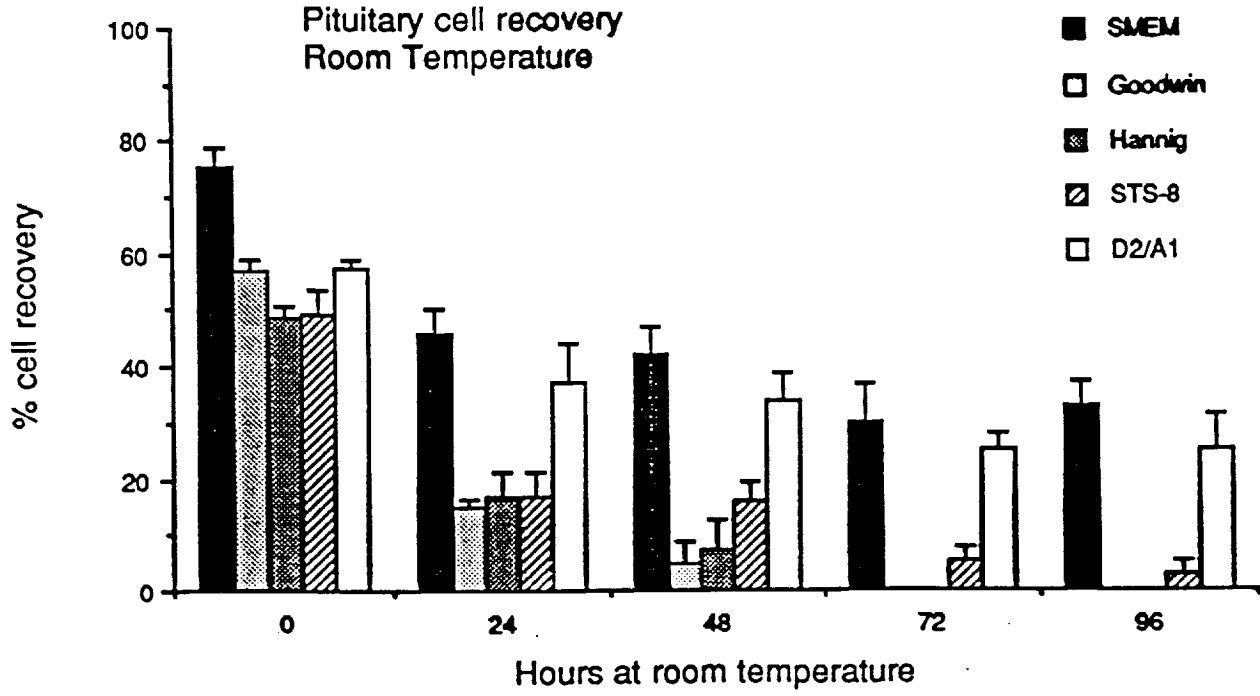




Figure 2

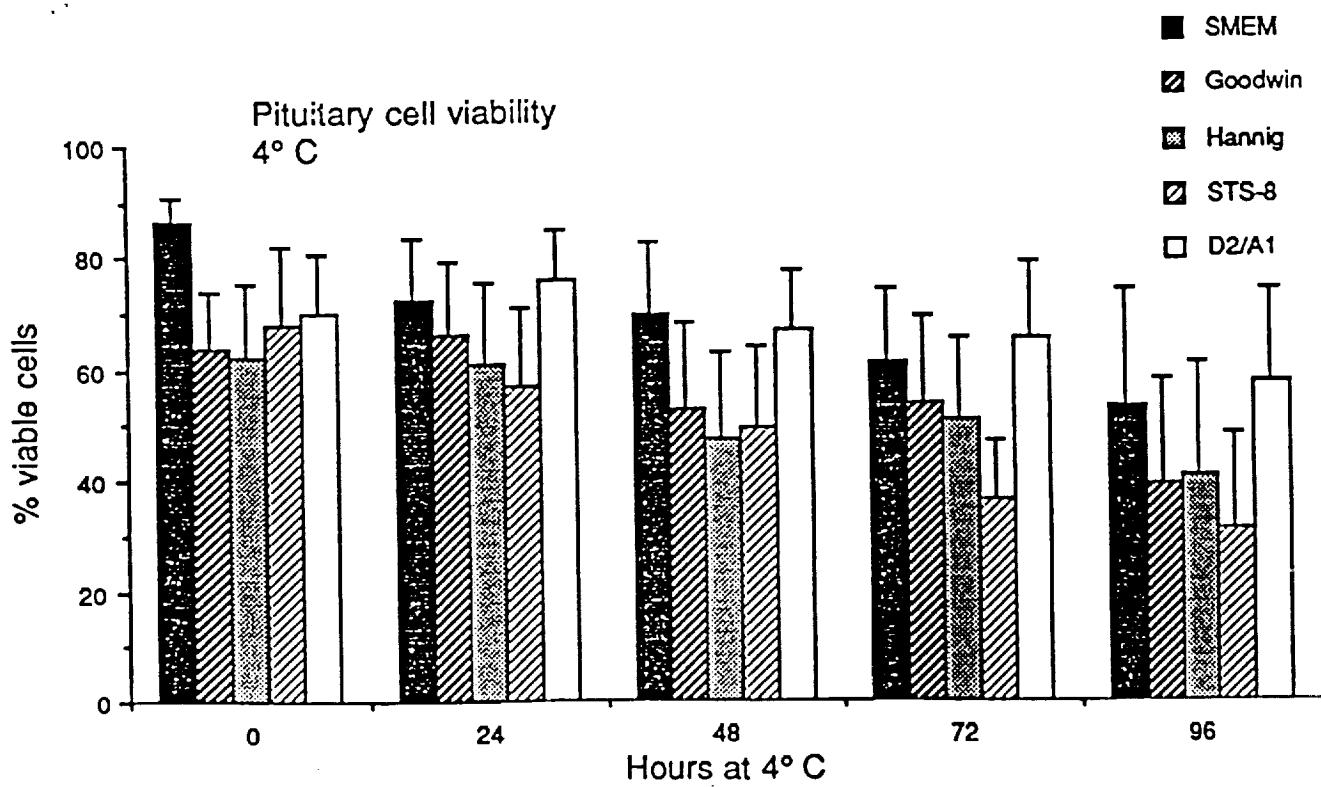
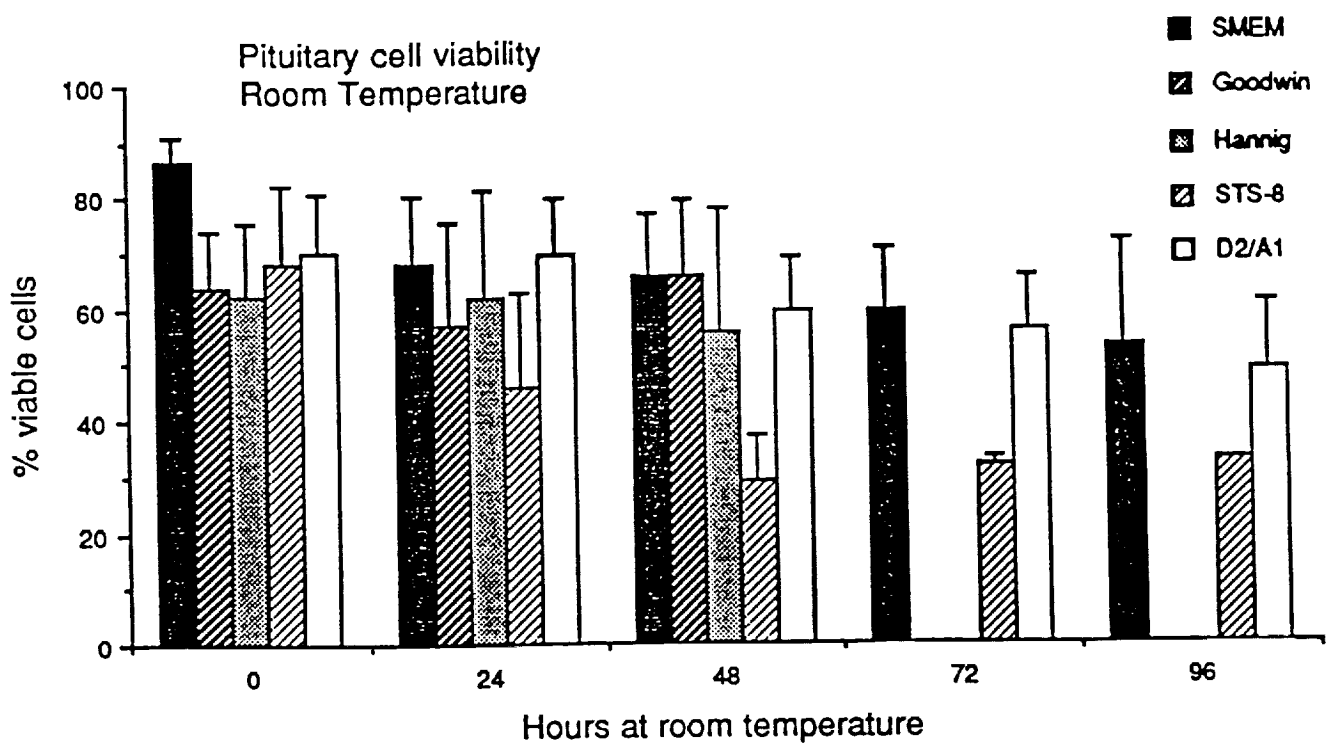


FIG 3

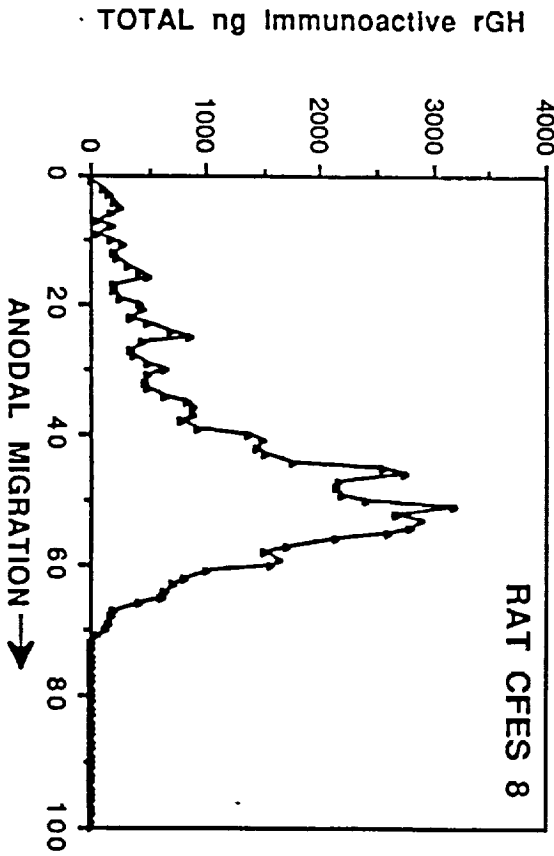
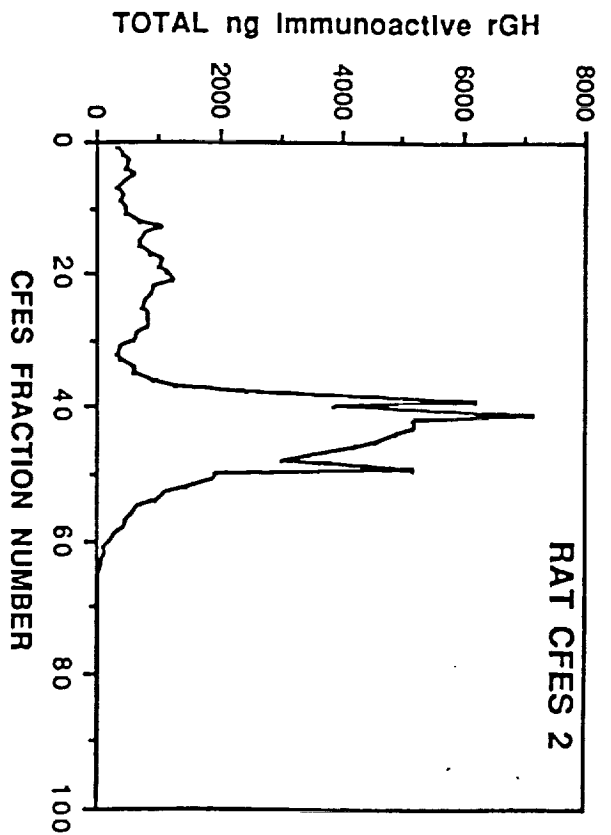
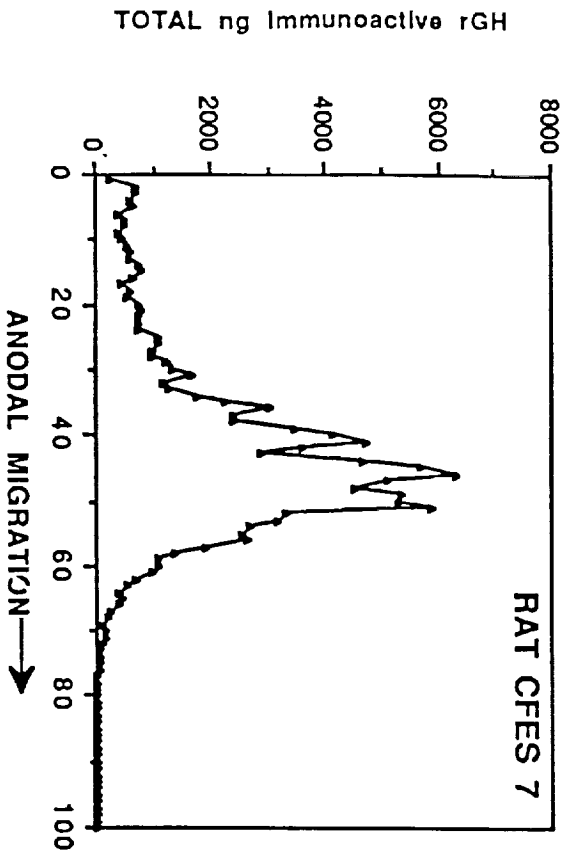
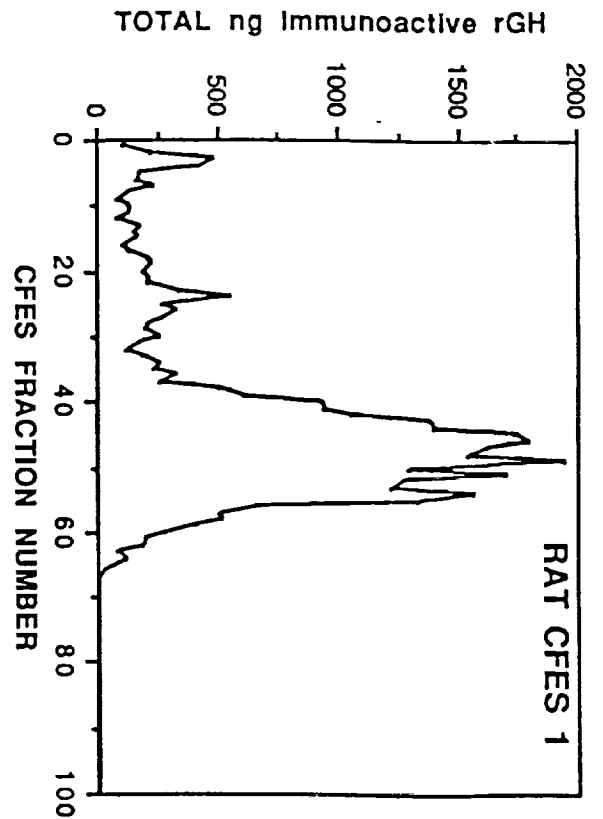


FIG. 4.

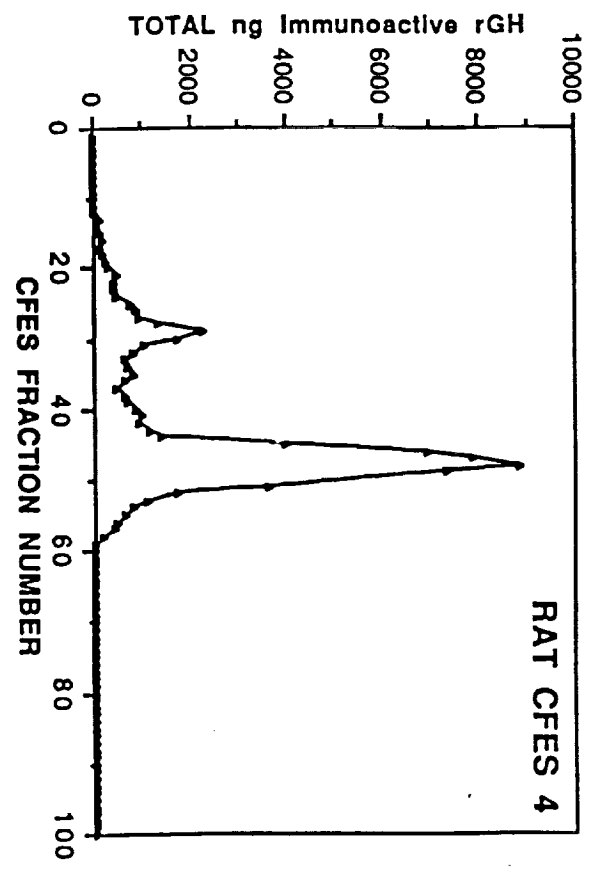
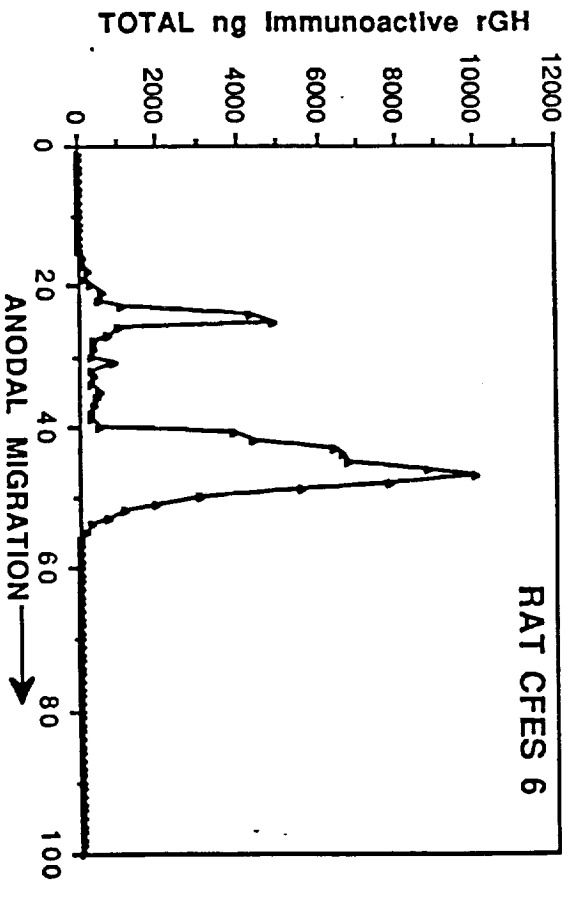
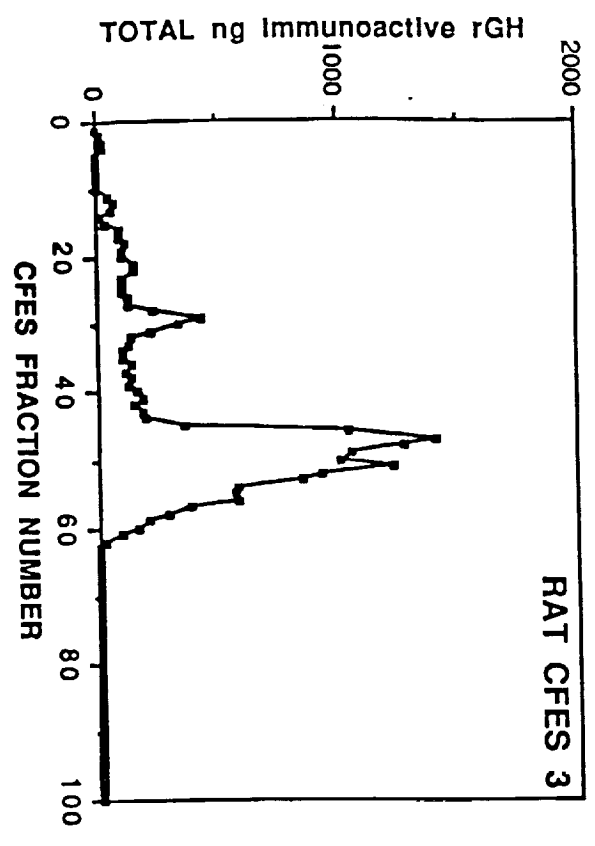
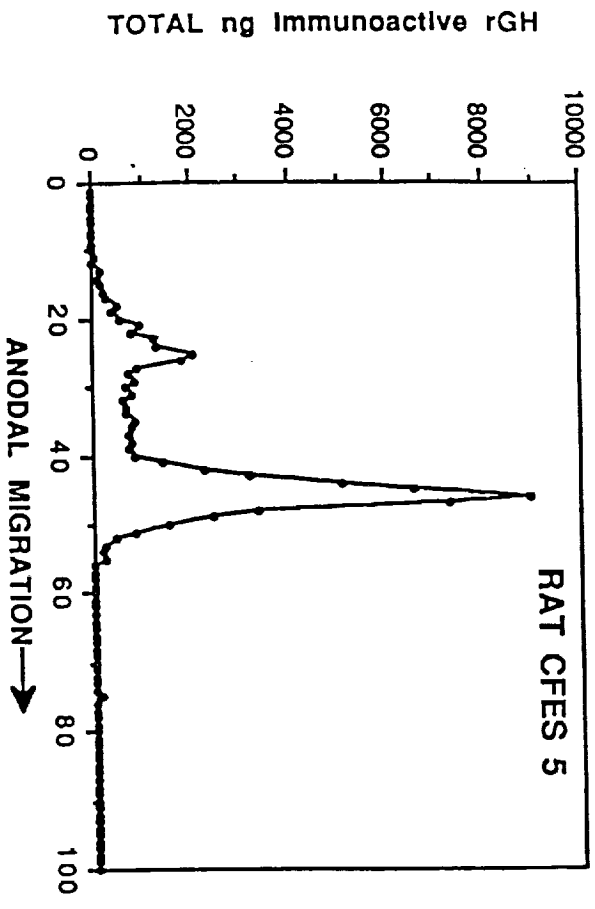
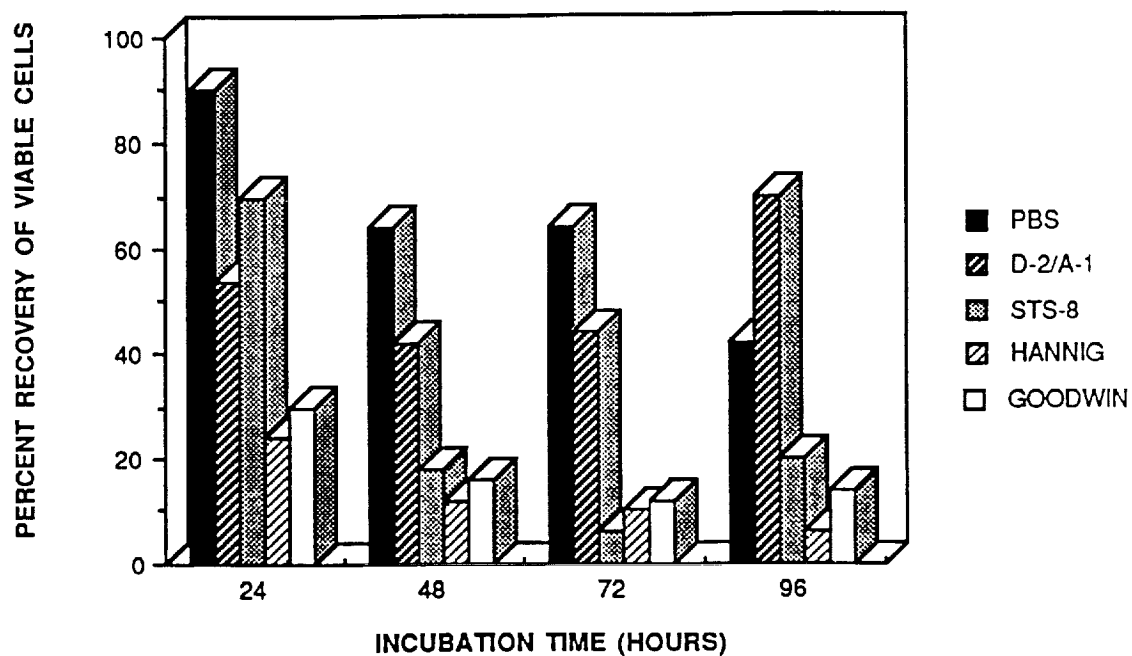


Figure 4

Figure 5

PERCENT RECOVERY OF VIABLE SPLENCYTES FROM ELECTROPHORESIS  
BUFFERS AFTER 24 TO 96 HOUR INCUBATION AT 4°C



PERCENT RECOVERY OF VIABLE SPLENCYTES FROM ELECTROPHORESIS  
BUFFERS AFTER 24 TO 96 HOURS INCUBATION AT 22°C

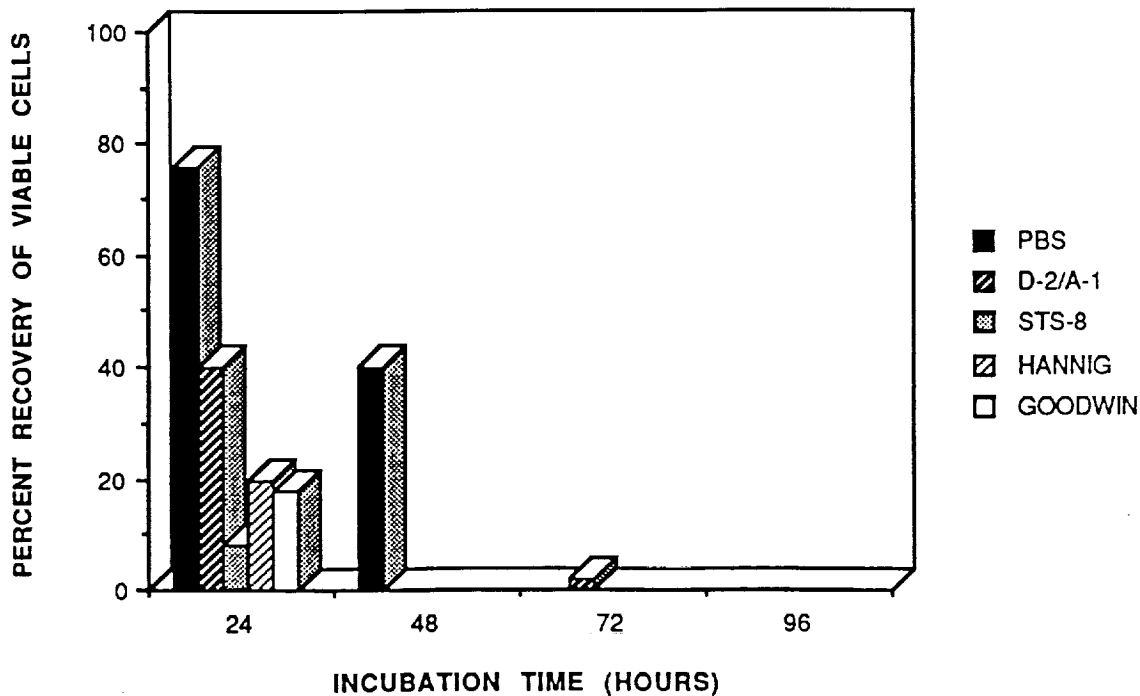
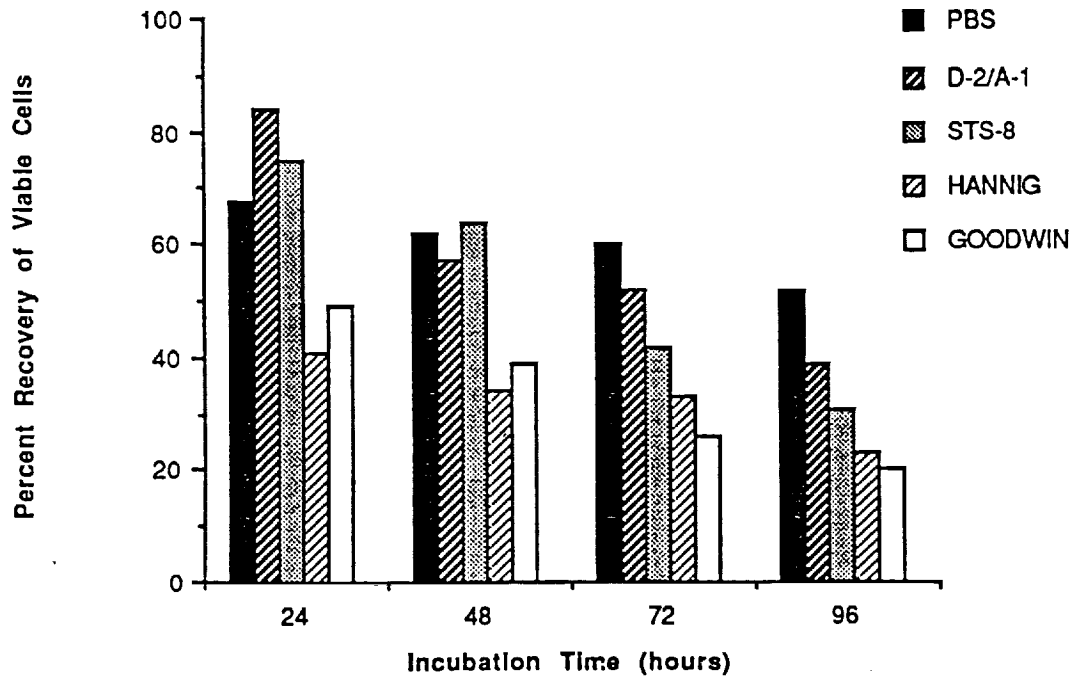


Figure 6

Percent Recovery of Viable Splenocytes from Electrophoresis Buffers after 24 to 96 Hours of Incubation at 4° C



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1. Survival of splenocytes after incubation in various electrophoresis buffers at 22°C for up to 96 hr.

Rat splenocytes ( $5 \times 10^6$  viable cells/ml) in approximately 20 mls, were incubated in 30 ml syringes, at 22° in various electrophoresis buffers for 24, 48, 72, and 96 hr. At each time beginning at time 0, a sample (0.1 - 0.5 ml) was taken and the cells counted with a hemocytometer. Trypan blue dye exclusion was used to determine cell viability. Dulbecco's phosphate buffered saline (PBS) was used as a control cell culture buffer. The electrophoresis buffers included modifications of D-2/A-1, STS-8, Hannig and Goodwin.

Results:

After 24 hrs in all buffers, there was a rapid decline in cell viability (Fig. 1). PBS gave the best cell recovery, 14% after 24 hrs. D-2/A-1 was similar. The other three buffers gave 4% or less in this time. At 72 hr cells were seen only in PBS and there the levels were 4%.

This experiment was done twice with similar results.

2. Survival of lymphocytes after incubation in various electrophoresis buffers at 4° for up to 96 hr.

Rat splenocytes ( $5 \times 10^6$  viable cells/ml), 20 mls, were incubated in 30 ml syringes at 4° for 24, 48, 72, or 96 hr. At each time beginning at time 0, samples were taken and the cells counted with a hemocytometer. Trypan blue dye exclusion was used to determine viability. Dulbecco's phosphate buffered saline

(PBS) was used as a control, cell culture buffer. The electrophoresis buffers were: D-2/A-1, STS-8, Hannig and Goodwin.

### Results:

After 24 hr the recoveries in PBS and D-2/A-1 were similar, approximately 77% (Fig. 2). Recovery in the other three buffers ranged from 40 to 60%. After 48 hr, the recovery declined to 65% in PBS, (best recovery); to 26% in Goodwin buffer, (worst recovery). Even after 96 hr, recovery in PBS was 46%, and in D-2/A-1, it was 30%. It was down to 10% in Goodwin buffer. This experiment was done 5 times.

### 3. Optimization of mitogen responses.

Splenocytes ( $1.25 \times 10^6$ /ml) were incubated at 100  $\mu$ l/well, 96 well plates, in RPMI-1640 medium at 37° in an 5% CO<sub>2</sub>, 95% air, humidified incubator. Cultures were stimulated with lipopolysaccharide (LPS) from salmonella typhimurium, dextran sulfate (DEX) or a combination of the two. Other cultures were stimulated with Concanavalin A, (Con A), or left unstimulated. Cells were stimulated over a range of concentrations, as shown. Con A is a T cell mitogen; LPS/Dex a B cell mitogen. <sup>3</sup>H-thymidine, (1  $\mu$ Ci/well, specific activity 6.7 Ci/mM) was added at 54 hrs of incubation. At 72 hr the cultures were harvested onto glass fiber filtermats with a Skatron harvester. The papers were counted with an LKB Beta plate liquid scintillation counter. The results shown are the average CPM (<sup>3</sup>H-thymidine incorporation) of triplicate cultures minus the incorporation of unstimulated cultures.

### Results:

. The optimum incorporation for Con A was 1  $\mu$ g/ml (Fig. 3a). With LPS/Dex the highest incorporation was seen with 10  $\mu$ g/ml each (Fig. 3b). The stimulation of T cells (Con A) was about 5 fold that of B cells (LPS/Dex).

#### 4. Electrophoretic separation of rat splenocytes on CFES unit PU7.

The concentration of rat splenocytes was adjusted to  $1.5 \times 10^7$  viable cells per ml in 10 ml of modified D-2/A-1 electrophoresis buffer. Modification of the original D-2/A-1 buffer consisted of lowering the salt concentration and adding sorbitol (pH = 7.3). Viability of the cell suspension, determined by trypan blue exclusion, was 93.3%. Cells were collected into a 20 ml syringe and kept at 4°C through the entire separation procedure. The sample was injected into the CFES column at 2.5 ml/hour and fractions collected at about 9.0 ml/hour with a residence time inside the column of about 6.0 minutes. A 90 minute cell separation followed a 30 minute equilibration of the column. One-hundred fractions were collected with approximately 12.8 ml total volume each. The total volume of cell suspension loaded was 3.83 ml ( $5.75 \times 10^7$  total cells). The fractions were centrifuged at 1000 rpm at 4°C and supernatant removed. The pellet was resuspended in approximately 0.5 ml of PBS with 2% FCS and cells counted on a hemacytometer. Cell recovery was low; only  $7.8 \times 10^6$  total cells were recovered (13.5%) but all cells were viable. The cells were divided equally for cell surface staining.

##### Results:

##### A. Cell distribution and cell recovery

All of the splenocytes were recovered in fractions 34 through 46 (Fig. 4a). The total cell recovery was low; only 13.5% of the cells loaded were recovered (4b). Most cells were found in fraction 41.

##### B. Separation of B and T lymphocytes

Each fraction was divided equally and one stained with antibody (anti CD5) to T cells, the other with antibody to B cells (Mark -1) (Fig. 5a). A fluorescent rat anti-mouse IgG (H+L), F(ab')<sub>2</sub> was used as a second antibody. Samples were analyzed by flow cytometry (Epics 753). T cells migrated in the



region of high electrophoretic mobility and B cells in the region of low electrophoretic mobility.

Unseparated samples contained approximately 80% T cells and 14% B cells (Fig 5b). After separation, the B lymphocytes were found mainly in fractions 37 to 39; e.g. Fraction 38 contained 65% B cells and 26% T cells. T cells were enriched in fractions 42 to 44. For example, fraction 43 contained 89% T cells, 6% B cells (Fig. 5). There was some overlap.

### Problems and Concerns

Cell aggregation at 4° is the major problem. We are currently testing the use of dispase (neutral protease) and DNAase to solve this problem. In preliminary experiments we have seen promising results.

### Plans for Year Two

1. Continue testing the effect of incubation conditions (buffers, temperature, time) on splenocyte function [i.e. DNA synthesis and interleukin-2 (IL-2)] release.
2. Cell electrophoresis will be continued and subsets of lymphocytes will be characterized.
3. Begin work with soluble receptors.
  - a. Test cell lines for presence of soluble IL-2 receptors.
  - b. Develop ELISA for detection of soluble IL-2 receptors.
4. Attempt to isolate plasma membranes from primary lymphocytes and lymphoid lines.