AD-A247 138

PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	91/11/29	~~Annual~~ Interim 90/11/1-91/10/31

4. TITLE AND SUBTITLE
A BIOLOGICAL MODEL OF THE EFFECTS OF TOXIC SUBSTANCES - Annual Technical Report No. 1

5. FUNDING NUMBERS
C
F49620-91-C-0012
61502F
3005
A1

6. AUTHOR(S)
Robert P. Tuckett, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Topical Testing, Inc.
421 Wakara Way, #130
Salt Lake City, UT 84108

8. PERFORMING ORGANIZATION REPORT NUMBER
01

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
USAF AFSC
Air Force Office of Scientific Research/NL
Bldg. 410
Bolling AFB
Washington, D.C. 20032-6448

10. SPONSORING/MONITORING AGENCY REPORT NUMBER
92 0004

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release
Distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

Due to the basic nature of military operations, it is sometimes necessary for Air Force personnel to be exposed to toxic chemicals in their work environment, either as a protracted low-level exposure or as a high-level, acute exposure. The Draize rabbit eye test for acute irritancy has come under severe criticism by the animal rights movement and has undergone legislative restrictions. Therefore, the tissue culture test being developed is likely to commercial as well as scientific benefit.

Progress by Topical Testing during the past year has been on a number of technical fronts including the development of data collection software and a method for delivery of microquantities of toxic chemicals to individual nerve cells in tissue culture. The neuronal cultures are now viable and reproducible, and their response to a variety of chemical stimuli has been recorded. Corneal epithelial cultures have been viable for a number of months. A number of strategies have been explored to expand the corneal epithelial culture and thus make it a viable commercial system.

In summary, Topical Testing has made substantial gains in the development of a commercial assay system, and next year will focus on testing the system's response to different classes of toxic chemicals.

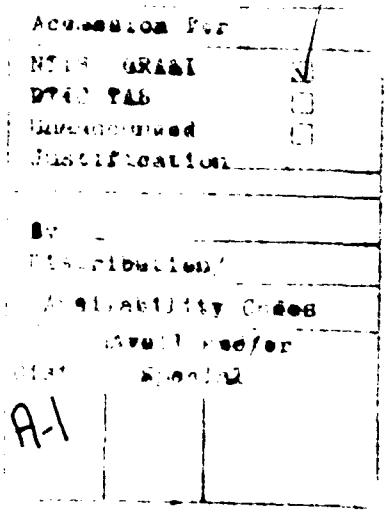
14. SUBJECT TERMS	15. NUMBER OF PAGES
Bioassay, Environmental toxins, Detoxification, Tussue Culture	61
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	U	U	SAR

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

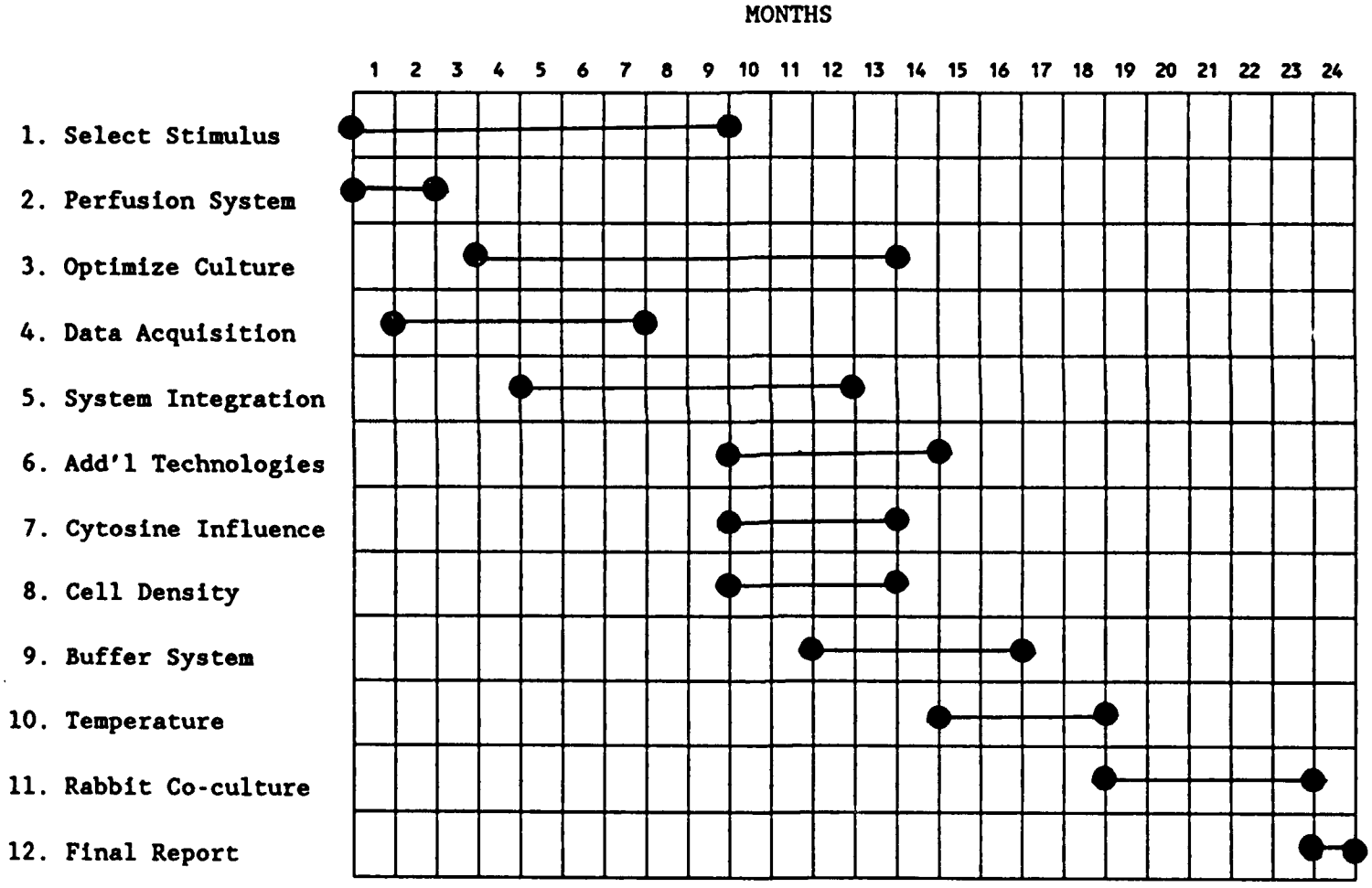
DMEM	Dulbecco's Modified Eagle's Medium
KGM	Keratinocyte Growth Medium
HBBS	Hank's Balanced Salt Solution
PC	Personal Computer
EMS	Expanded Memory System
DAS	Data Acquisition System
MHz	Mega Hertz
kHz	kilo Hertz
Mbyte	Mega byte
µM	micro Molar
mM	milli Molar
µl	micro liter
µm	micro meter



LIST OF TABLES

LIST OF FIGURES

TABLE I. ORIGINAL SCHEDULE OF SPECIFIC OBJECTIVES



SPECIFIC OBJECTIVES OF THE RESEARCH

The contract is to continue research and development of a prototype physiological model for monitoring the effects of toxic chemicals and further elucidate the mechanisms of action of toxic chemicals on the peripheral sensory nervous system. Specific objectives follow:

1. *Selection of a standard pollution stimulus.* Because of the complexity and variety of chemical species that have been tested with the in vivo Draize eye test and because of the necessity of comparing Topical Testing's in vitro model with the in vivo data, definition of a standard pollutent stimulus will require testing of a wide variety of compounds. The objective will be to compare the response of neurons grown in culture with and without enrichment with corneal epithelium. The working hypothesis is that since neurons that innervate cornea are specifically reactive to noxious stimuli, there will be a shift in the response properties of the neurons in co-culture with corneal epithelium such that a significantly greater number of neurons have nociceptive properties and thus are responsive to irritant and noxious chemical stimuli. (The membrane properties of the neurons in both cultures will also be compared.)

2. *Develop a perfusion system which can rinse away the chemical stimulus in between stimuli.* A perfusion system will be implemented which minimizes the electrical noise produced by the pump and optimizes methods of sterilizing and cleaning the system after each day's use.

3. *Evaluate how long cultures must grow before testing.* Determination of the minimal length of time neurons must be in culture before testing involves evaluating the health of the neuron as measured by parameters such as the resting action potential and stability of recording and also the specificity of response to chemical stimuli.

4. *Evaluate different data acquisition systems (DAS) to determine which best suits current application.* DAS systems will be evaluated for speed, price, and compatibility with IBM PC compatible computer systems. The compatibility of the data acquisition systems with high speed PC computer systems will also be evaluated as well as the specifications of computer systems which can sample and display the data at high resolution. (It is estimated that 10 kHz will be an adequate sample rate to maintain data integrity.)

5. *Continue development of action potential recognition and data summary software.* Software development is a critical aspect of commercial efficiency. Means for recording the signals at high frequency for extended periods of time. Stimulus selection, handling and analysis of data, summarization, quality graphics, and data backup and storage are some major issues to be investigated.

6. *Make improvements in the total integrated system.* There are a number of aspects to the total system (e.g., chemical delivery, recording, vibration, isolation) which when combined can provide a more efficient and commercially viable assay system.

7. *Evaluate current clamp, voltage clamp and fluorescent technologies.* Current clamp is the most robust method for measuring membrane properties. Unlike voltage clamp, data integrity is maintained independent of cell geometry. Hence, current clamp will be used to analyze membrane properties during the current contract. In addition, the feasibility of using voltage clamp in future research efforts will be investigated. Also, the ability of newly developed technology of using fluorescent probes to monitor changes in ion flow will also be reported. The voltage clamp and fluorescent probe technologies might have advantages in answering questions in specific experimental situations.

8. *Determine whether cell inhibitor(s) significantly influence the qualitative response to the chemical stimulus in co-culture.* Neurons, when grown in isolation, contain a mixture of fibroblasts, and when grown in co-culture with epithelium contain the epithelium plus fibroblasts. The purpose of this task is to determine whether the use of cell inhibitors to slow fibroblast proliferation will have a significant impact on epithelial growth, and if so to investigate other methods of isolating neurons from fibroblasts in order to obtain a purer co-culture of neurons enriched with epithelium.

9. *Determine whether cell density qualitatively affects chemical stimulus response.* The possibility that density of neurons plated affects neuronal response properties will be tested by plating neurons at different densities looking for differences in response to electrical and chemical stimuli.

10. *Determine whether the buffer system significantly influences the qualitative response to chemical stimulus.* The recording media must contain a buffer to maintain pH within the physiological range. The buffer system used in the tissue culture growth media is bicarbonate, and the cells are maintained in 5% CO_2 in the incubator atmosphere. When the cells are removed from the incubator for recording, there is a choice between using HEPES buffer (e.g., Hank's solution) and continuing to use bicarbonate (e.g., Earl's solution). Bicarbonate is technically more difficult to use because it requires a continual bubbling with CO_2 and monitoring of the pH to maintain medium within physiological limits. Experiments will investigate whether the economically more efficient HEPES buffer is an adequate buffering system for Topical Testing's technology.

11. *Determine whether perfusion solution temperature qualitatively influences response to the chemical stimulus.* While being grown in the incubator, the tissue culture is maintained near core temperature (36-37°C). Historically, many, if not the majority, of tissue culture studies have recorded at room temperature. However, there is a possibility that the response to chemicals will be qualitatively different when the culture temperature is maintained near body temperature. This possibility will be investigated.

12. *Determine whether rabbit neurons have a qualitatively similar response as rat neurons.* Since the traditional Draize test is performed in rabbit, it will eventually be necessary to compare rat co-cultures with those obtained in rabbit. This task will will investigate on a preliminary basis the feasibility of comparing rabbit and rat responses in tissue culture.

The GANTT Chart in Table I shows the original anticipated scheduling of tasks during the contract period. Table II summarizes the tasks performed to date, and Table III shows the anticipated scheduling of tasks over the remaining contract period.

RESULTS TO DATE

Introduction

The purpose of this project is to design a commercial assay system with which to assay for pain and irritation using an *in vitro* test analogous to the Draize eye test in which neurons from the trigeminal ganglion (which normally innervates the ocular surface) are grown in culture with the epithelium from the ocular surface and thus establish an *in vitro* assay system for irritation. The response of an individual neuron to a chemical stimulus will be recorded and its activation will be suggestive that the stimulus has irritant properties (Fig. 1).

As shown in Figure 2, the experiment can be divided into several components: First, the tissue culture model has been extensively and continually modified with the goal of designing methods which will economically and reproducibly generate co-cultures with corneal epithelium (Figs. 3,4). The chemical delivery has been designed using microperfusion technology (Figs. 5,7,8). The setup itself has undergone refinement and includes a number of components including the recording system, the tissue culture environment (e.g., perfusion system), and the recording media. The data acquisition software has undergone continual improvement (Fig. 6). Lastly, the physiological data is reviewed (Figs. 9-21).

Tissue Culture

Trigeminal Neuron Culture

Several important technical changes have been implemented that have substantially improved the neural dissociation.

As established in phase I research, it has been possible to grow neurons in culture with a collagen substrate. In early phase II, with the advice of Dr. Alcayaga, the dissociation procedure was modified to relatively large cell bodies. Larger cells are suggestive that the dissociation process is more gentle and is yielding more viable cultures.

The initial protocol called for the ganglion tissue to be passed back and forth through a small-tipped glass pipette to break up the tissue for dissociation. A gentler technique is now being used in which after isolation each ganglion is partially pulled apart with jeweler's forceps and then placed on ice in HBSS (Ca^{++} and Mg^{++} free). After all the ganglia are isolated, they are placed in dissociation solution. With this technique, a greater variety of cell sizes are being obtained. [Larger cells suggest that dissociation procedure is more gentle (Dr. Alcayaga, personal communication)].

It was discovered that a different dissociation protocol was necessary for fetal animals than for newborn/adult animals. Hence, up until delivery, sensory neurons in the trigeminal ganglia have little collagen. Hence, it is not necessary to add collagenase to the dissociation protocol, and the ganglia are digested with trypsin in HBSS. In the older animals, collagenase and DNAse aid

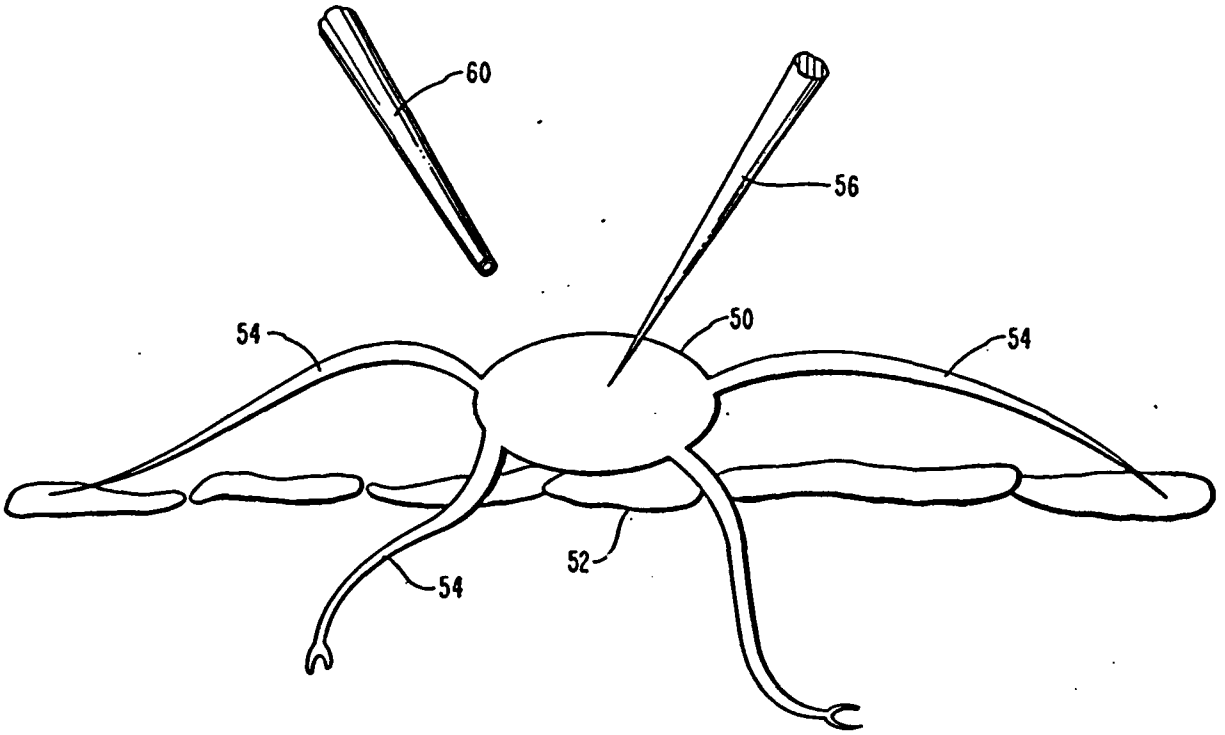


FIGURE 1. An idealized drawing of the biological model being developed. After dissociation, the neurons (50) from the trigeminal ganglion are plated onto the epithelium and grow out neural processes (54) which establish contact with the epithelium. A microelectrode (56) penetrates the cell body (50) of a neuron. Microquantities of the toxic chemical to be assayed are then pressure injected in the local vacinity of the neuron through a micropipette (60) and the response of the neuron to the potentially irritant chemicals is recorded.

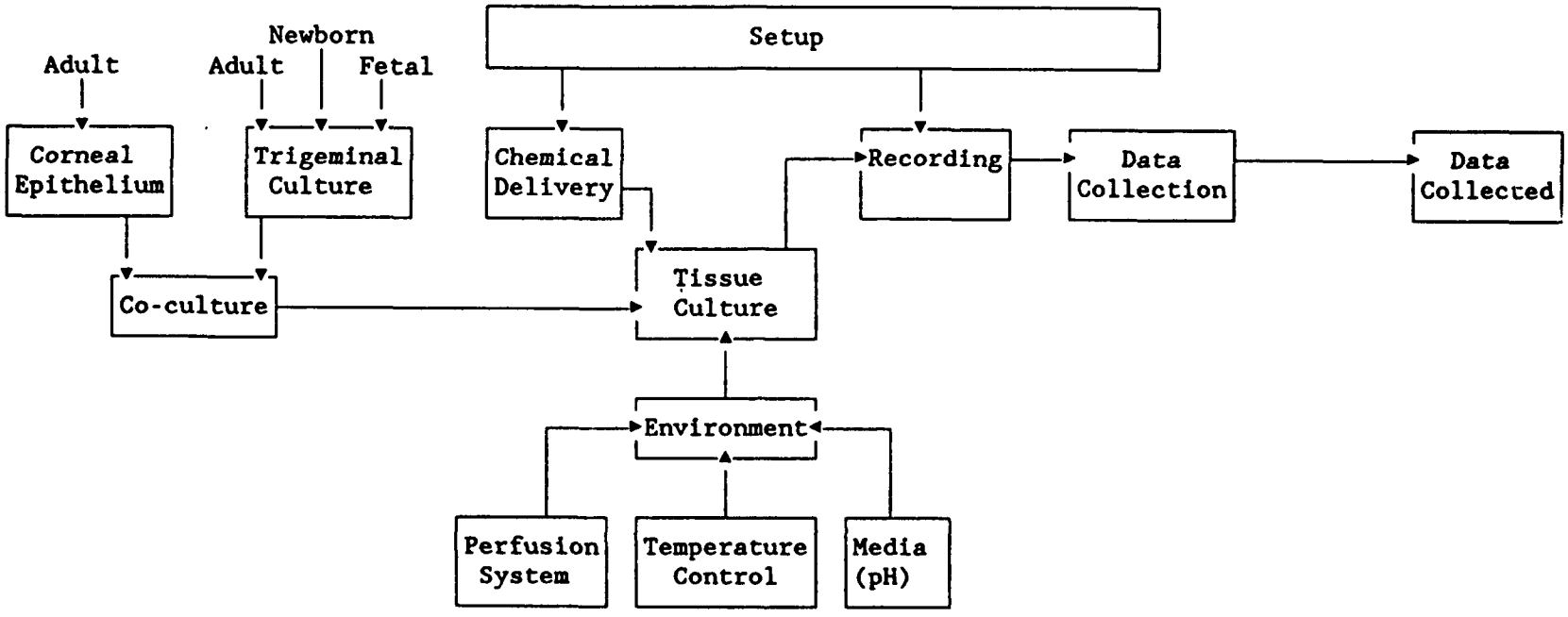


Figure 2. Outline of the areas of focus during the first year's development. The assay system is complex, with a variety of components to be optimized for the eventual development of a commercial bioassay for pain and irritation.

in the dissociation process. These changes have substantially improved the yield in all age groups. Currently, timed-pregnant rats are ordered so that the culturing of neurons is timed to be from 1-2 day old animals. To date, neurons have been successfully cultured from rats varying in age from 17 day fetal to fully grown animals of up to 500 gm.

Tissue Culture Media. Two types of media have been used: (1) DMEM (Dulbecco's Modified Eagle's Medium) with inactivated horse serum, and (2) Keratinocyte Growth Medium (KGM, from Clonetics) which is a standard medium for growing epithelial cultures. It has been found that neurons grow effectively in both media; however, there are differences in neuronal development.

The DMEM medium produces neuronal cell bodies that are rounded and clear under phase microscopy. Fibroblasts tend to proliferate in the DMEM; however, it is possible to identify and record from neurons among the fibroblasts. As mentioned in the previous report, cytosine has been used to inhibit fibroblast proliferation but are currently are not being used. If necessary, fibroblast outgrowth can be minimized (1) with cell inhibitors, (2) by cell density separation techniques, or (3) by using KGM media (see below). The neurons produce normal looking action potentials after 2½-3 days and continue to have a normal response.

About 6-8 days post-culture, some neurons begin to die. Other neurons remain, and responses from the remaining neurons are being recorded for up to 15-20 days in culture. (In earlier experiments, neurons were grown and normal responses were recorded up to two months in culture.) Alternative causes of the cell death are currently being investigated (see below). As a practical matter, cell death of neurons after 8-10 days may not be an important issue in the current assay system because there a time window of 5-7 days for recording is sufficient for assay purposes.

Keratinocyte Growth Media (KGM) has different effects on the neural growth than DMEM. Perhaps most striking is that in 100% KGM, the cell bodies of the neurons have an unusual shape which is "lumpy" in contrast to the smooth circular (or oval) shape of neurons grown in DMEM. Axons are often thick and easily observed under the microscope. In addition, fibroblast growth is inhibited in KGM and the neuronal cell bodies do not tend to migrate together to form "pseudoganglia" as they do with DMEM (see next paragraph). So far our impression is that the cells begin to die at about the same time (8-10 days) as in DMEM; but as with DMEM, a number of neurons persist. As the percentage of KGM-to-DMEM is lowered, the cells begin to take on more of a "normal" appearance as seen in 100% DMEM. Eighty-percent KGM to 20% DMEM appears to be an optimal percentage for neural growth when grown with fibroblasts. Currently this 80/20 media ratio is being used in our neuron cultures.

In the neuron-epithelial cell co-cultures (see below), the neurons appear to grow well in 100% KGM. Hence, the co-cultures are currently being grown in 100% KGM to take advantage of the ability of KGM to inhibit fibroblast proliferation while enhancing epithelial proliferation.

Neuron Migration. After about 5-6 days in DMEM, the neurons tend to migrate in the culture dish and form into clumps which have the appearance of "pseudoganglia" and they are with axons and appear to innervate nearby tissue. Migration is a disadvantage because it is more difficult to identify individual

neurons for recording. Migration is minimized by lowering the density of neurons that are plated in the dish and by using KGM media.

Fibroblast Proliferation. The fibroblasts tend to "take over" the longer the neurons are in culture, and it is our impression that the fibroblasts tend to grow in close association and will often contribute to a connective layer over the neuronal cell body which can make it difficult to record from the neuron.

Different methods for inhibiting fibroblast growth have been investigated: (1) It was found that the neurons grow well in pure DMEM and will grow well in DMEM + KGM media [KGM is a growth media for keratinocytes]. As KGM concentration was increased, an inhibition of fibroblast growth was observed. When 100% KGM was used, the fibroblasts died and also the neurons would die. Upon close inspection of neurons in early culture (<1.5 days), neurons usually grow in close association with fibroblasts. Hence, a conclusion from these preliminary studies is that neurons might not grow in culture without a supporting "target" cell.

When the neurons are added to epithelial cultures in 100% KGM, the neurons will grow. Hence, the working assumption is either that epithelium allows fibroblasts to grow in 100% KGM and the surviving fibroblasts allow the neurons to grow, or that the neurons can grow with epithelium in the absence of fibroblasts.

It is important to minimize the background of fibroblasts because they tend to obscure the neurons and make recording somewhat more difficult in older cultures and because they tend to compete with epithelial cells in co-culture. Hence, cell isolation procedures are currently being investigated to separate the neurons from fibroblasts.

One technique for isolating neurons is differential adhesion. Immediately after dissociation of the trigeminal ganglion, fibroblasts have a greater tendency to stick to the bottom of a plastic petri dish (Corning) than do neurons. After plating the dissociated cells onto a petri dish, waiting 10 min and then repipetting the neurons, fibroblast numbers were lower. However, as discussed before, if the number of fibroblasts drops too low, the neuron culture does not survive. By replating the fibroblast-neuron mixture 2-3 times, it is possible to remove an even greater percentage of the fibroblasts.

Experiments are now beginning to optimize procedures for purifying the neuronal dissociation and neurons are being added to epithelial cultures in 100% KGM. The working assumption is that trigeminal neurons will grow with epithelium in the absence of fibroblasts. If fibroblasts are necessary, the ratios of fibroblasts-to-neurons will be minimized before plating on previously established epithelial cultures. Work is planned with our consultant, Dr. K. English, to determine whether more refined cell separation techniques would be beneficial in the current application.

Neuron Survival in Culture. Although some neurons survive for up to eight weeks in culture, a high percentage die after about 8-10 days. There are at least two general alternative mechanisms for cell death: (1) Working Hypothesis. Different types of sensory neurons are predisposed to seek out and innervate specific types of target tissues. If these targets are not available in the co-culture, the neurons will die. Alternative Hypothesis. Something about the tissue culture environment is detremental to many of the neurons. To test the alternative hypothesis, several factors that contribute to the tissue culture

environment have been investigated, including the dissociation process (see above), media (see above) and substrate.

To investigate the possibility that the substrate influences cell survival, different types of plastic have been used with no dramatic differences in neuronal survival. In phase I, no significant improvement in neuronal growth was seen when matragel or laminin (Collaborative Research) was substituted for collagen. During the past few months, different collagens have been tested from different suppliers (collagen type III from calf skin, Sigma #C-3511; collagen type I from calf skin, Sigma #C-9791; collagen type I from rat skin, Boehringer Mannheim biochemicals #1063-812). Experiments suggested that type I collagen from rat skin gave the most consistent neuronal cultures. Experiments were then conducted to look at the effects of collagen dilution and density on neural growth. A plastic surface (polystyrene) was tested but found that the epithelia cells did not appear to adhere as well as to collagen.

The filters in the water purification system were changed and the water was checked for contamination using conductance measurements.

As an additional test for optimal neuronal survival, different media were investigated for optimal growth that was compatible with both neuronal and epithelial growth (see above). In addition, the dissociation process was optimized for different aged animals (see above).

These efforts have resulted in a stable neuronal culture that progresses through a reproducible series of stages in culture. Hence, the working hypothesis is that death of neurons in culture is a normal process related to the co-culture environment and that the neurons that remain in culture are those that have contained a prespecified class of target cell which provides trophic influences necessary for continued development. Experiments are planned for next year to test whether neuronal responses to chemicals are significantly influenced by the age of the culture.

Epithelial Culture

It is more difficult to obtain viable corneal epithelial cultures from older animals because it is more difficult to get the cells to adhere to the substrate and begin to proliferate and because once a culture is established, the rate of proliferation appeared to be significantly less than in younger animals. On the other hand, epithelium from young animals appeared to proliferate more rapidly but the corneas are significantly smaller than older animals, thus limiting the initial number of cells available for culture in each animal. Because it is important to have a high plating density (see below), corneal size is an important variable to be considered. As a consequence, young adult animals seem to be the best choice as a donor and corneal epithelium is currently being harvested from young adult rats (about 200-250 gm).

Although it has been possible to grow corneal epithelial cultures since the phase I research, corneal epithelial cultures have been rather inconsistent in that in some experiments the cells would not attach to the substrate; in others, the cells would attach but would not begin to proliferate for 5-12 days in culture, and in other cultures the cells would quickly adhere to the substrate and begin to proliferate within 24 hrs.

Talks with other investigators (e.g., K. English, D. Forbes) combined with our experience suggests that corneal epithelium is more difficult to grow the cutaneous epithelium. Unlike neuronal cultures, the epithelial cultures require a high initial density of viable cells before the culture will begin to multiply and divide. At present, it is uncertain whether the cells must be in actual physical contact with one another or just be within a certain distance, nor is it known what the total number of cells in the culture must be. Initial experience suggests that if the cells quickly attach (within 1 hr) to the substrate and if the density of neurons is great enough, then the culture will begin to proliferate within a day. In some cultures although the epithelial cells adhered, the cells did not begin to proliferate for several (3-4) days but then began to proliferate satisfactorily and grow toward confluence. To date, no clear reason has been found why proliferation is delayed in some cultures. However, in general the sooner proliferation begins the more rapid, uniform and healthy the cultures appear.

Because a "critical mass" of epithelial cells is required for proliferation and because the rat cornea is a small tissue, it is clear that directly plating the epithelium onto a 35 mm dish (which requires about 6 corneas/dish) is not an optimal design for a commercial assay. Hence, alternative means are being investigated to extend the epithelial harvest into a maximal number of cultures.

Expanding Epithelial Cultures. Several strategies are being investigated to expand epithelial cultures. One technique for expanding cultures in a commercial assay system would be to replate new cultures from seed cultures for several cycles. Topical Testing has demonstrated the feasibility of growing the corneal epithelium to confluence in 35 mm dishes. The cells have then been redissociated using trypsin and replated, and successful 1-to-4 expansions have been obtained. Hence, because about 6 corneas are required to originate a culture in a 35 mm dish, then a 1-to-4 dilution will yield a dish for every 1.5 corneas that were originally harvested. Furthermore, the replating process can be continued through several cycles. We have demonstrated that the cycle can be repeated at least three times. However, there are several disadvantages to this approach: (1) reaching confluence can require a prolonged time period (up to 3-4 weeks) and KGM media is expensive; (2) often when epithelial cultures are replated, some cultures don't take and hence the yield decreases; and (3) perhaps most importantly, with each replating there is increased likelihood the epithelial cells might tend to lose some characteristics that are important for the co-culture assay. A modification of the replating technique would be to expand the epithelial culture to confluence in 35 mm dishes and then replate the cells at high density into several 35 mm petri dishes using 10 mm cloning cylinders to restrict cell migration until after the cells have attached and the colony has begun to proliferate into several areas using cloning cylinders (Fig. 3).

There are reasonable arguments to restrict the epithelial culture to a relatively small area of the 35 mm petri dish: (a) To reduce the possibility of deterioration with time, the cultures are recorded for a maximum of half a day; hence, there is no need to grow hundreds or thousands of neurons to study over a large area of each petri dish. (b) Because the volume of the chemical stimulus is very small, it only reaches a small restricted area around the neuron being studied, it is quickly diluted by the surrounding recording media and is swept away by the perfusion system (Fig. 7). In addition, by first studying neurons closest to the exit of the perfusion system, stimulus chemicals will always be carried away from neurons which have not been studied (Fig. 7). (c) Because of

the physical dimensions of the condensor and the base plate of the microscopes, it is necessary to "angle" the recording and perfusion micropipettes over the edge of the petri dish (Figs. 7,8) in order to simultaneously record and pressure inject single neurons. Therefore, the edges of the petri dish cannot be studied. Hence, it is possible to restrict the study to a small area in the center of the dish and hence minimize the surface area of epithelial growth necessary for study in each tissue culture plate.

Concentrating and Restricting Epithelial Growth. Two techniques have been investigated for increasing the density of epithelial cells that are plated and for restricting the area of epithelial growth. One technique is to grow the cells on small pieces of coverslips in multiwell dishes and then transferring the coverslip to a 35 mm petri dish prior to recording (Fig. 3). The other method is to position a sterile template (i.e., cloning cylinder) in the center of a 35 mm petri dish to restrict the exposed surface area. These techniques have two common advantages: First, the initial number of epithelial cells ("critical mass" as discussed above) necessary to seed the surface area is smaller because the surface area is smaller and hence a high initial density of corneal cells can be obtained with a much lower total number of cells. Our initial estimates that there will be at least an order of magnitude savings in the number of donors required. Hence, instead of inquiring about 6 rat corneas per dish less than 1 cornea will be necessary. Secondly, once the epithelium is established, it will grow to confluence more quickly than in a larger dish. These two protocols are discussed in more detail below.

Small pieces of coverslips were cut or broken, autoclaved and placed in multiwell dishes (Corning, cell wells #25820, 24 well, flat bottom, 16 mm diam., tissue culture treated, polystyrene, sterile). Three types of coverslips were tested: glass (Bellco #1943, microslip cover circles, No. 1 thickness, 12 mm diameter), a plastic coverslip (THEMANOX #T-3055-13 in Intermountain Scientific Catalog, 13 mm diam.), and a common plastic slip (Scientific Produces #M6100, Dispo slips, clear, noncurling, unbreakable plastic, thickness 0.17-.25 mm, 22 x 22 mm). Each was coated with collagen as in the neuronal cultures. Unexpectedly, the dissociated epithelium adhered best to the Scientific Products' coverslip. It was found that during plating a significant number of epithelial cells flow off the cover glass slip and onto the surrounding "moat-like" area. Our fabrication consultant (Mr. Evans) has custom designed small teflon cloning cylinders to insert in the multiwell dishes and restrain the epithelial cells from flowing off the coverslip and thus maximize epithelial cell density. Epithelial cultures are growing much better in both the teflon templates and commercially available glass cloning cylinders (see below) now that they are being precleaned in Chromerge solution (Monostat Inc., chromic-sulfuric acid). (In addition, some of the initial templates were made out of material that was sold by a local supplier as teflon, but dissolved in Chromerge!) Hence, Chromerge cleaning appears to be an important technical procedure both because it likely removes potentially toxic materials and is an assay for teflon imitations.

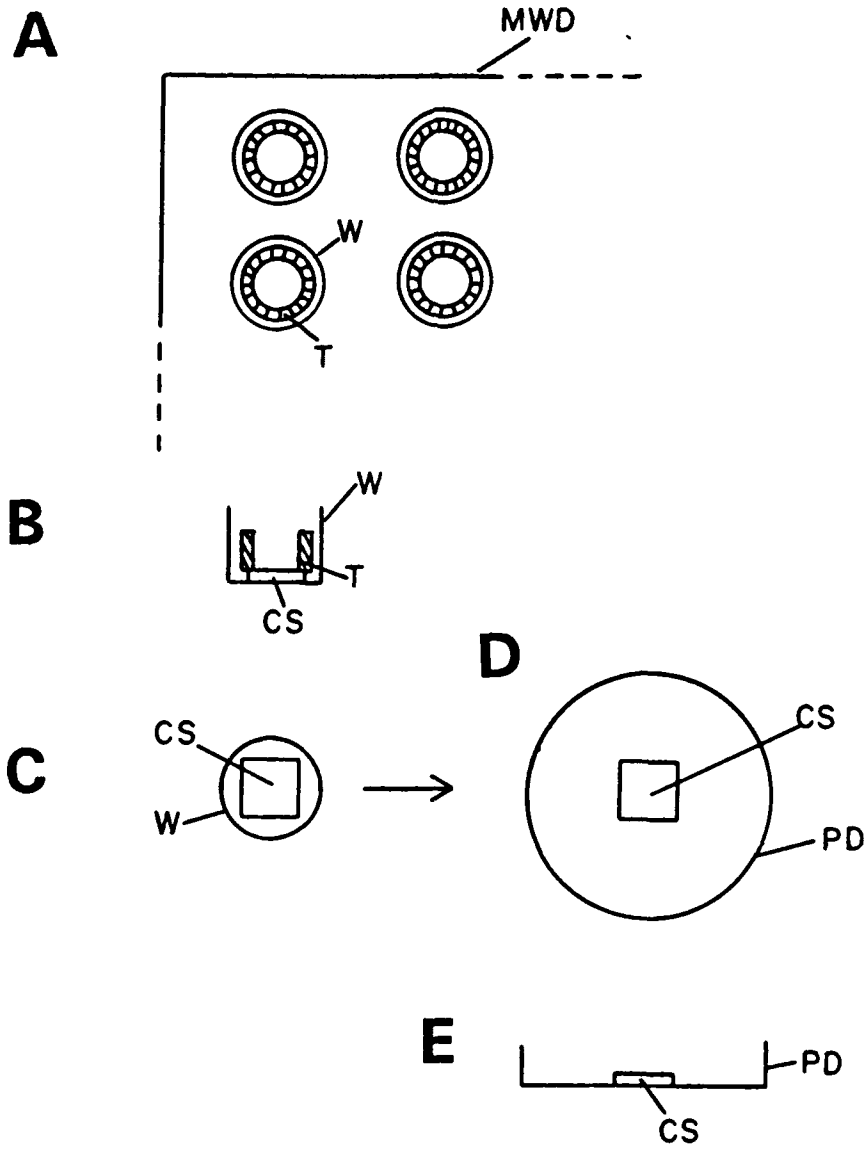


FIGURE 3. Illustration of method for expanding the epithelial culture. The epithelium is dissociated and then plated into multiwell dishes (MWD). A: illustrates a section from a multiwell dish. Each well contains a ring template (T) constructed of teflon by our fabrication specialist (Mr. Evans). B: A rectangular piece of plastic coverslip (CS) is centered at the bottom of each multiwell dish and the teflon template is placed on top of the cover slip. Dissociated epithelial cells are then plated onto the coverslip. The template has three major purposes: First, it reduces the area onto which the epithelium is plated, thus minimizing the absolute number of cells necessary to obtain a critical density... (*figure legend is continued on next page*)

FIGURE 3 continued. ...so that the epithelium will begin to proliferate (see text). Secondly, after the template is removed there is some added area on the coverslip into which the epithelium can continue to grow, thus allowing the epithelium to continue to grow without maturing and dying. [When the epithelium grows until it is restricted so that it can no longer multiply and divide, it becomes "old" and begins to degenerate. Hence, having an added area for the epithelium to grow into provides an added time period for continued health of the culture.] Thirdly, the template on top of the coverslip provides a barrier so that the epithelial cells will not flow from the coverslip and onto the surrounding surrounding "moat" around the coverslip in the well. Thus, cells are not lost that otherwise would remain in the well after the coverslip is removed for study. C: As mentioned above, after the epithelium is plated and begins to proliferate, neurons are plated onto the coverslip. After the neurons adhere, the template is removed. Thus the epithelium and neurons can mature at the same time on a small piece of plastic. D: After appropriate time interval, the coverslip is transferred to a standard 35 mm petri dish (PD) for recording in the experimental setup (see Figs. 5 and 7). E: shows a lateral view of the petri dish with the coverslip lying at the bottom. Our initial experiments have shown that once the coverslip is placed in the petri dish with recording media, the coverslip becomes mechanically stable on the bottom of the dish within about 5 min so that the neurons can be impaled and stable recordings can be obtained.

The use of templates might have an additional advantage. The epithelium adheres to the substrate best if there is a minimal amount of media in the dish when the cells are first plated. However, the volume of media must be increased within a few hours of plating in order to minimize the changes in osmolarity and pH that might occur over longer time periods. Initial experiments suggest that adding the media to the area outside the template and then allowing the media to slowly flow under the template from all directions is less disruptive to cell attachment that directly dropping the media into the chamber (Fig. 4B).

In addition to adding templates to multiwell dishes, templates have been inserted in 35 mm petri dishes (Corning #25000, 35 mm tissue culture dish, polystyrene, sterile, was the 35 mm dish used in all experiments after collagen coating), and it was found that the glass cloning cylinders (Bellco #2090; 8 mm o.d., 8 mm height or 10 mm o.d., 10 mm height; chemically inert borosilicate glass, ends of rings round to ensure a tight seal with surface of sulture vessel) tend to move around and hence scrape the epithelial layer off the bottom of the dish. Subsequent experiments using a series of tight-fitting "donut-shaped teflon inserts did not slip and hence cell damage minimized change. By drilling two appropriately spaced holes about 2-3 mm deep in the top of the teflon insert, it is possible to insert jeweler's forceps in the holes and lift the templates vertically out of the dish without disturbing the cell layers.

Hence, dissociated epithelial cells can be grown in multiwell dishes on coverslips and then transfer them to 35 mm dishes before recording or grown directly on 35 mm dishes. As mentioned above, the corneal epithelial cells appear to adhere more effectively to plastic coverslips than directly to the 35 mm dish (both surfaces are collagen coated); therefore, it is likely that plastic coverslips will be used for both multiwell and 35 mm dishes.

After the dissociated epithelium is plated, it tends to adhere to a number of points on the dish and if the "mass" of cells is great enough, it will spread out in what appears to be a single layer until it reaches the border of the dish or the edge of the cover glass slip. After it reaches its boundaries, the cells tend to stop multiplying, and after 2-3 days the cells begin to die. Hence, it is important to keep the epithelial cells in a growth cycle during the tissue culture process. Our technique for maintaining epithelial cell growth is to remove the template (Fig. 3B) that is restricting the epithelial growth when the epithelial cells have grown almost upto its borders. The protocol is to plate neurons after the epithelial culture is well established, but while there is space around the epithelium for the neurons to attach to the collagen. Experience suggests that the neurons will form strong attachments within 4-6 hrs, at which time the inner template will be removed and the epithelium will continue to grow out to the boundaries of the new template while the neurons are maturing and establishing functional connections (in about 3 days), at which time their response to toxic chemicals can be measured as described elsewhere.

Instead of complete dissociation, an alternative method for growing corneal epithelium is to place small fragments of the epithelial layer in the culture dish (Fig. 4). Donna Forbes (personal communication) has found that corneal epithelial fragments will attach and grow <u>if</u> the fragment is oriented such that its inner surface attaches to the substrate. She said that her procedure is to peel off a small piece of cornea and orient it in a "small drop" of media on the collagen surface. The small drop keeps the fragment oriented correctly, such that it will adhere and quickly begin to multiply. In initial experiments using the Forbes' technique, it was difficult to maintain the fluid level in the

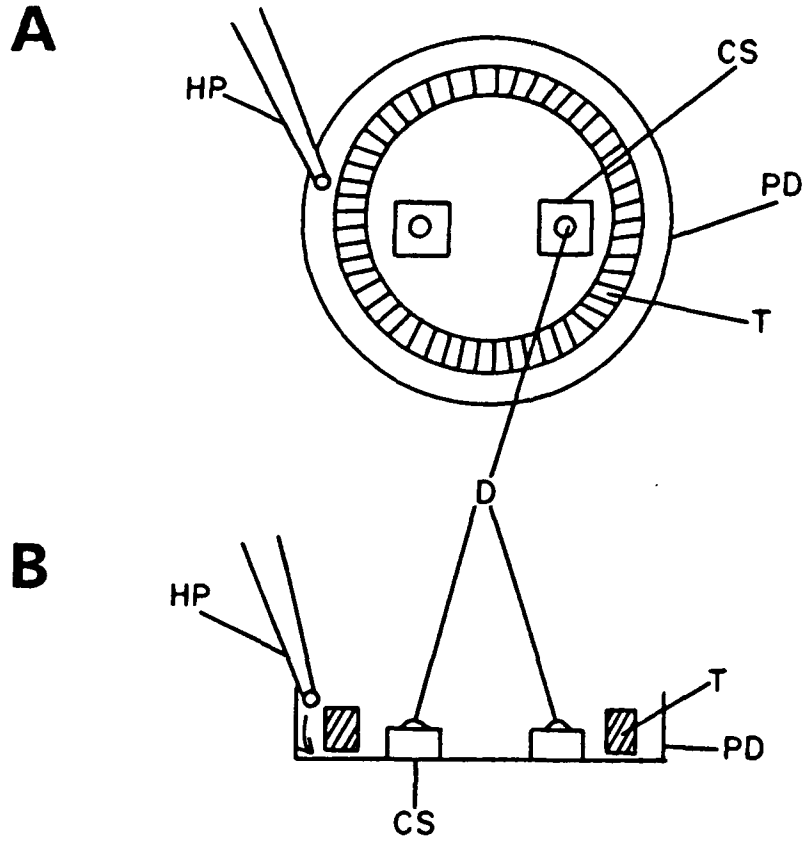


FIGURE 4. Culturing corneal epithelium. A: Technique for plating fragments of corneal epithelium (FC) into droplets of media (D) placed on pieces of collagen-coated plastic coverslip (CS) which have been placed in a 35 mm petri dish (PD) prior to collagen coating. After a few hours in the incubator, media is slowly added to the outside of a template (T) ring from a hand-held micropipettor (HP) which allows the media to uniformly distribute itself around the culture dish. B: A side view showing the droplets of media (D) resting on the cover glass slips and more media being pipetted behind the template (T) and flowing around and under the template to gently cover the bottom of the petri dish.

droplet. [The dehydration rate in the laminar flow hood is high. Her experiments were in Wisconsin and hence her laboratory might be higher in humidity.] When the fluid level was increased, the fragment tended to float and not adhere to the dish. To enhance the chances of firm attachment, her protocol called for the fragments to remain in small droplets of media for several hours before adding more media. However, in our experiments even after a few hours, there still was a tendency for the fragment to detach when new media was added.

To compensate for these problems, in future experiments each 35 mm dish containing droplets of media will be kept in the incubator until an epithelia in the dish fragment is ready for transfer and then it will quickly be transferred to the hood, the epithelium transferred, and returned to the incubator as quickly as possible. In addition, the room humidity will be monitored and if low, a sterilized ultrasonic humidifier will be used to compensate. These procedures should minimize decreases in droplet volume which could cause increases in osmolarity beyond physiological limits. In addition, techniques will be designed to more gently and gradually add media and thus minimize detachment. These strategies including making minichambers in template from which the media would slowly flow into the larger chamber and using smaller pipette volumes to gradually add the media (Fig. 4B).

Summary

Although co-cultures are currently growing and are being recorded, the technique for producing co-cultures requires refinement before reaching a commercial stage: There must be a way (1) to focus the epithelial culture to the area of the dish to be recorded from, (2) to obtain a uniform epithelial culture, (3) to restrict the area of neuronal plating so that corresponds to the epithelial culture (plate the neurons so that they attach to the area of neuron growth but not to other areas of the dish), (4) to have a means for the epithelium to continue to grow after the neurons are plated so that the epithelium does not age before the neuronal response is measured, and (5) to have a means for minimizing the number of fibroblasts in the neuronal culture. As discussed above, research in these areas is currently underway and will continue during the next contract year.

Perfusion System

Before recording, the culture solution (see elsewhere) is replaced with recording media. Initially, experiments used a Hank's (Sigma) solution with an additional 10-15 mM HEPES to buffer pH. The cells appeared to respond normally for at least 4 hours.

As illustrated in Figure 5, a perfusion system was constructed in which Hank's solution was circulated using a roller pump to control the flow rate. A second tube of larger diameter was installed in the roller pump and connected to a second tube which was inserted in the bath. By raising and lowering the suction tube, it is possible to control the fluid level. In order to electrically isolate the perfusion system from the recording setup, "drip isolators" are used which isolate the recording setup from ground. Such a system is currently being evaluated. Currently, experiments are running at room temperature. The effects of temperature on physiological response will be tested next year.

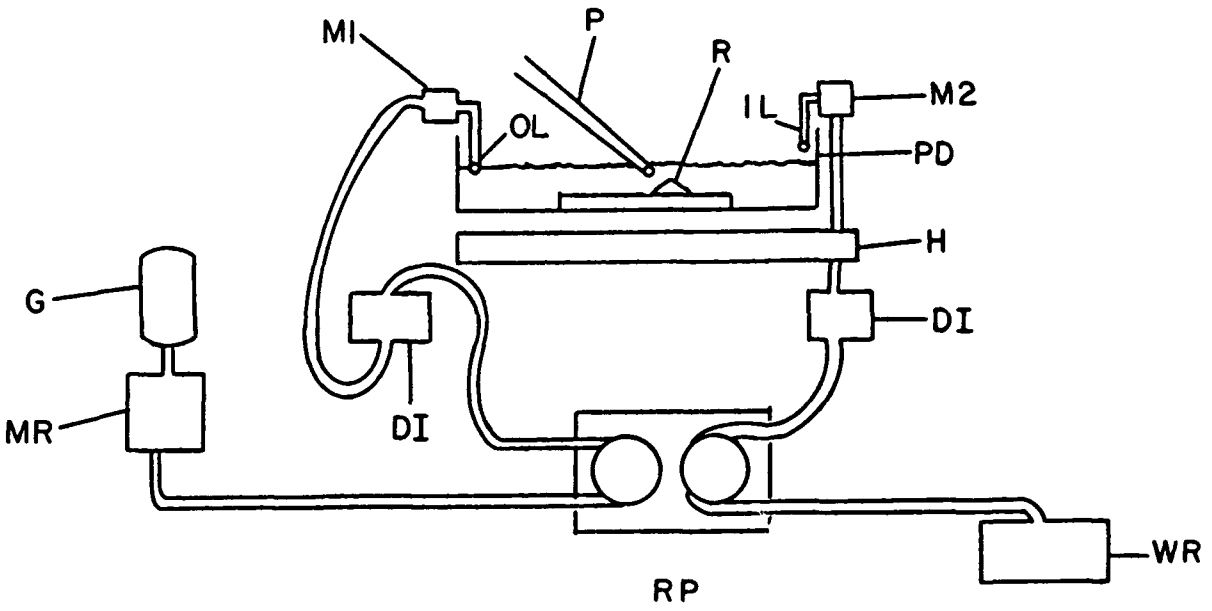


FIGURE 5. Perfusion system design. The petri dish (PD, 35 mm) containing the tissue culture (see Figs. 2-4) is placed in a plastic holder that fits in the base plate of the microscope (see phase I Final Report). The inlet (IL) and outlet (OL) of the perfusion system are positioned as close to the edges of the petri dish as possible in order to leave room for pressure injection (PI) and recording (R) electrodes. Since the flow of perfusion fluid is from the inlet toward the outlet, neurons closest to the... (*figure legend is continued on next page*)

FIGURE 5 continued. ...outlet are recorded from (R) first in order to minimize the possibility that the chemicals released from the pressure injection pipette (P) can reach neurons that have not yet been studied. Because the tubing from the outlet is larger than that to the inlet and because the inlet and outlet tubings are connected to the same roller pump (RP), the maximum rate of flow out of the petri dish is always greater than the inlet rate. Hence, the level of media in the dish is controlled by raising or lowering the level of the outlet port with a miniaturized manipulator (M1). A similar manipulator regulates the height of the inlet port (M2). The fluid flow through the inlet and outlet is electrically isolated from the tissue culture recording by drip isolators (DI) which create an air gap and thus eliminate ground loops and electrical noise generated by the roller pump. The recording media is held in a reservoir (MR) before being sucked into the roller pump. Gas (G) from a pressurized cylinder is constantly being bubbled into the media. The gas is 100% O_2 when Hank's solution is used with a HEPES buffer as a recording media and is 95% O_2, 5% CO_2 when Earle's solution is being used with a bicarbonate buffer (see text). The exit flow is collected in a waste reservoir (WR). During the second year of the contract (see text), a heating block (aluminum) will be installed to warm the temperature of the recording media before it reaches the petri dish and to directly heat the petri dish. The temperature of the media will be feedback controlled to a preset temperature through a thermocouple placed in the tissue culture (not shown).

A comparison of neuronal response in Hank's (HEPES) and Earl's (bicarbonate) buffered solutions has shown no significant differences to date. More extensive testing is planned for the coming year.

Data Acquisition and Analysis Software

For phase II development, a new software package was written in a combination of Microsoft, QUICKBASIC, and C-languages. C software allowed maximal speed of data handling and hardward control, QUICKBASIC is compiled and hence faster than BASIC and has flexibility in menu construction, writing equations and plotting data. The data acquisition system was changed from Keithley to Metrabyte. The advantage of the Metrabyte system was greater speed. Also, the entire board was on a bus in the computer system, making it less bulky and easier to manage. The design goal was to be able to sample the membrane voltage at a 10 kHz rate. Sampling at a 10 kHz rate gives a maximum frequency response of 5 kHz which was judged to be adequate for obtaining nondistorted waveforms of the membrane response to both room temperature and core temperature preparations. With the use of C programming, it was possible to control the board with use of the fast computer system (PC320386, 33 MHz). It was possible to sample both the stimulus and the membrane response at 10 kHz and the memory limitations of the computer did not exceed the 100 msec required to record the cell's response to electrical stimuli. On the other hand, sampling for much longer time periods was required (up to several minutes) in order to track the response of a neuron following chemical exposure. It was found that due to the limitations of computer memory (640K total) the program could only run for a maximum of 3 sec sampling at the 10 kHz rate. It was decided that alternative strategies must be explored. First, the possibility of using expanded or extended memory was investigated. However, the EMS controller necessary to access expanded memory required too much overhead time to allow sampling at a 10 kHz rate. In addition, it was found necessary to sample 3 channels simultaneously (membrane response, current and pressure waveform). Hence, a total of 30 kHz needed to be stored for extended periods. An alternative was to store the data on a digital tape or a VCR recording system and perhaps computer control of the tape recorder could be implemented. However, after evaluating the Axon system hardware and software which has the capability of recording directly onto disk, it was determined that likely our system could exceed the specifications of the Axon system and could record onto the hard disk using a DOS command structure.

As shown in Figure 6, the data acquisition software for sampling membrane voltage, pressure injection, and constant current stimuli has three major components. First is the run option with which the data is collected, second is a review section, and thirdly a backup onto cassette tapes. Each one of these components will be discussed separately.

I. In the run mode, the computer stores an information file containing some general information about the target tissue and about the neuron culture. With each experimental run, the program also stores the protocol being used for stimulation. For example, in the constant current stimulus mode the type of hyperpolarizing or depolarizing pulse is recorded in the information file along with a second file containing the sampled data. For depolarization, both 5 msec and 50 msec duration pulses are used to determine threshold. For suprathreshold, only the wider duration pulse is used looking to determine whether there is

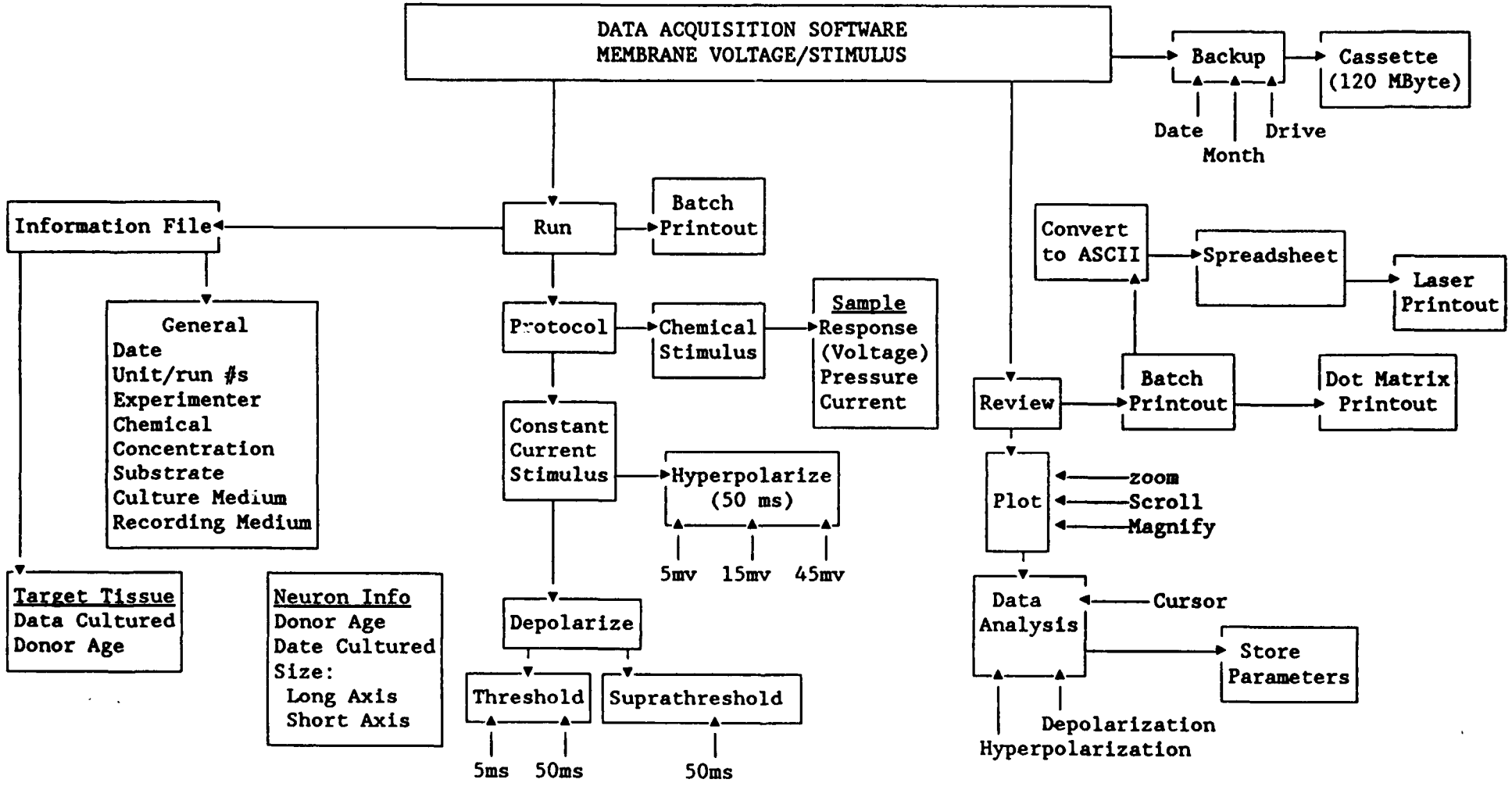


FIGURE 6. Logical structure of data acquisition and analysis program. See text for detailed description.

multiple spikes generated by the constant current pulse. For hyperpolarizing, all pulses are 50 msec in duration, and a series of increased voltages are used. For both the current stimulation and the chemical stimulation, pulses are generated independently of the computer by a electrical stimulator. A long-term goal of the project which is beyond the phase II tasks and objectives is to implement a program with computer control of the stimulation parameters.

II. The review section of the program replots the data and has the ability to zoom in or out in time to review large segments of data and then magnify segments of interest, as illustrated in Figure 12. The program can scroll through the record and amplify the amplitude of the membrane response or the stimulus waveform. For analysis, a cursor can be placed on the response to measure precisely the voltages and the timing of the membrane response. There is also a section for curve fitting and determining the parameters of the response to the chemical stimuli.

III. Because of the large amount of data digitized, it is necessary to use a large hard disk (80 Mbytes) and to backup the hard disk regularly. A cassette backup system has been found to be ideal for this purpose, and there are options to backup daily, by the month, or by the whole drive.

A "snapshot" capability is being developed so that the data can be reviewed and records and portions of records can be selected for review and printout. In addition, a "batch print" capability is being established so that all the selected runs can be printed sequentially, along with the information files associated with each run. Formats are currently being developed so that the batch print can be directly output to a laser quality printout using QUATROPRO for formating the print process. A procedure to quickly analyze cell membrane characteristics is being implemented. The program was a curve fitting and pattern recognition routine to recognize the important characteristics of the cell response to depolarizing and hyperpolarizing currents.

In addition, an action potential recognition strategy is being investigated with which the time between action potentials (interspike interval) can be measured and firing frequencies calculated after drug injection. This software will give added tools with which to evaluate the ability of toxic chemicals to excite sensory neurons in culture. With this additional software, the rate at which action potentials are generated over extended periods of time can be analyzed, as well as changes in membrane potenital.

Because of their length, printouts of software code have not been included in this Annual Report. Final versions will be included in next year's Final Report at the end of the contract period, and current listings are available on request.

System Integration

Vibration Isolation. The setup is currently located on a solid basement floor and the microscope rests on a 1/4 inch iron plate which is isolated from the experimental table by four tennis balls. This arrangement appears to be adequate for our current working environment; however, a professional vibration isolation table is recommended for a commercial setup.

Micromanipulator Technology. Initially, a Kopf fluid microdrive was evaluated, but it was unstable, was nonlinear in its movement, and exhibited hysteresis to forward and backward movement. A Narashige manipulator with motorized morote control was found to be adequate in its specifications but had been in use for several years and its remote control was not reliable. There were reports from other investigators at the Medical Center that Narashige fluid drives are often not stable (tended to creep over time). A piezoelectric microdrive (Burleigh, #6000) is currently being used. It has performed well, it is stable with little or no observable creep, and has high resolution (0.1 μm) over an extended range (>1 cm). It has a remote control with a variable speed control and has either programmable steps or continuous movement. The only significant limitation is that it is rather heavy and bulky. As a consequence, it has been difficult to find a base micromanipulator on which to mount it. A manual Narashige micromanipulator was evaluated, but it was difficult to keep the gear and clamping mechanisms from loosening and it required constant adjustment. A Pryor manual micromanipulator (Stoelting, #55102) was tested, but it did not have an adequate return spring and could not be adequately adjusted to hold the Burleigh. A Stoelting micromanipulator (MM33, #55133) is currently working adequately. However, a heavier manipulator such as a Huxley would probably be recommended for a permanent commercial installation of the Burleigh microdrive. For example, Burleigh has recently introduced a specialty microdrive (Burleigh Instruments, #PCS-1000) for in vitro recording. Alternatively, there are new manipulators with a piezo motor with similar specifications and is within the same price range as the Burley drive, plus it has 3-axes of remote control movement which is a significant upgrade from the Burleigh drive (Fine Scientific Tools, Marzhauser #25200).

Recording System. A newer model WPI electrometer was installed to provide increased capacitance compensation. In addition, the shielding between the link between the head stage and the mount to the manipulator were redesigned to reduce noise and ground loops, as well as the mount between the electrode holder and the WPI head stage.

Chemical Delivery

Because the purpose of the system is to assay toxic compounds, a chemical delivery system is being designed that will minimize the amount of chemical handled by the workers (Fig. 8) and isolate chemical delivered to the cell under study. As illustrated in Figure 7, using pressure injection technology it is possible to load micropipettes with small volumes of solution (<1.0 μl) and then pressure inject minute volumes (picoliters) in the vacinity of the cell under study.

Chemical Delivery Micropipette. In consultation with Dr. Alcayaga, who is an expert in delivery of chemicals with micropressure injection, a standard glass

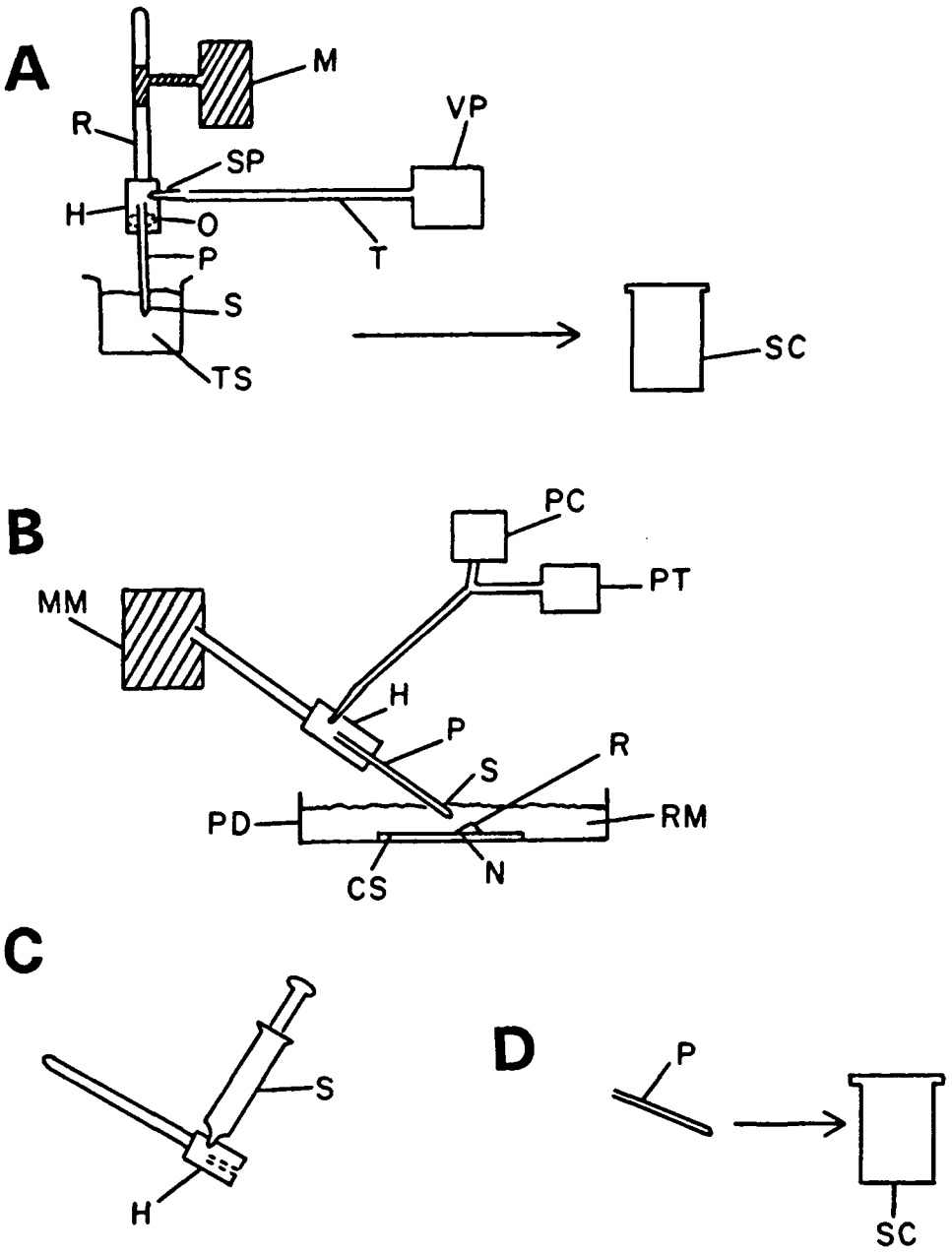


FIGURE 7. Illustration of the chemical delivery technology. As illustrated in Figure 6B, the pressure injection pipette (P) is mounted in a holder (H) and the sample (S) of test solution is sucked into the tip of the pipette using a vacuum pump (VP). The pipette with holder is transferred to the experimental setup, is mounted in a micromanipulator (MM), and under microscopic observation is positioned approximately 100 μm away from the neuron (N) being recorded (R). A: gives a more detailed view than Figure 6 of the components of the pressure injection hardware and electronics. The controller is capable to producing either a constant vacuum... (*figure legend continued on next page*)

FIGURE 7 continued. ...through a vacuum pump (VC) and sensitive vacuum regulator (VR) that was specifically fabricated to compensate for fluid leaking from the pipette tip. Leakage can occur due to hydrostatic pressure (see HP in B) or due to increased size of the tip of the pipette (see C). On the other hand, the controller is capable of producing a constant pressure head through a pressure tank (T2) of inert gas (100% N_2) connected to a specially fabricated sensitive regulator with which the capillary pressure can be compensated. The controller also produces a step pulse and pressure which causes the chemical to be pushed out of the tip of the pipette. This is accomplished through a second gas canister of inert gas (100% N_2) connected to a second regulator and timing of the pressure pulse is controlled by a stimulator (S). The pressure wave from the controller is monitored by a pressure transducer (PT) and the signal from the transducer is sent to the computer. B: shows a simplified diagrammatic sketch of the pressure forces at work during steady state. The capillary pressure tends to suck fluid up into the pipette from the solution. The amount of capillary pressure is dependent on the characteristics of the solvent placed in the microelectrode. On the other hand, the hydrostatic pressure (HP) is produced by the gravitational pull of the fluid column and tends to push the fluid out of the pipette. C: The amount of leakage out of the pipette is influenced by the tip diameter. Panel 1 shows a diagrammatic sketch of thin pipette whose tip is almost beyond view of the light microscope level. Such pipette tips were found to be of a disadvantage for these experiments because of the pressure wave produced by pushing the fluid out of the tip caused a mechanical artifact during recording. In addition, the small tips easily are clogged. The second drawing illustrates a relatively larger pipette tip. Current experiments use a tip about 4-9 μm in diameter. The tips are pulled using the Sutter puller which is programmed to produce tips of uniform diameter. The third example is illustrative of tips of greater diameter (12-15 μm and greater) through which there can be a continual flow of solution. Tactically it is possible to use a tip of larger diameter and to control the leakage out of the pipette with vacuum and by recording first from cells closest to the exit of the perfusion system as explained in Figure 5.

capillary was selected of the type used in patch clamp (AM Systems, 1.5 mm o.d., #7052). The Sutter puller (Sutter Instruments, #P-87) was used to pull tips of various sizes. Unlike the recording electrodes, the tip sizes of micropressure injection pipettes are within the limits of light microscopy, and hence it is possible to directly measure dip diameters using a compound microscope with a calibrated micrometer eyepiece (450x). Initially, tip sizes of 0.5-1.5 μm internal diameter were used; however, it was found that during pressure injection, the pressure wave exiting the micropipette was frequently sufficient to mechanically dislodge the recording micropipette from the neuronal cell body. Furthermore, optimal positioning of the pressure injection pipette was examined in control experiments. It was found that with a small tip not only was the exit velocity high causing a significant pressure wave, but also the path traveled by the dye was narrow and hence pressure injection electrode had to be positioned distantly from the cell being recorded. This issue is important for a commercial assay system because optimal recording efficiency requires that the experimenter be able to see the outline of the pressure injection pipette while viewing (400x) the cell being injected. If the pressure injection pipette can not be viewed under high magnification, it cannot be accurately aimed at the cell under study and a significant amount of time is spent shifting between 400x and 200x to insure the proper orientation of the electrode. [One cannot switch magnifications while recording because the mechanical vibration will dislodge the recording pipette from the neuron.] In addition, it is important to be able to view the tip of the pressure injection pipette tip to insure that it has not become partially clogged by crystalization or protein buildup.

For these reasons, very small tips were not used. Further experimentation suggested that tip diameters of 4-8 μm appeared to be more optimal because of (1) minimal tip clogging, (2) a wider envelope of chemical delivery, and (3) a lower exit velocity such that the force pressure wave reaching the cell was low enough that there was no evidence of tissue injury, recording electrode dislodgement, or mechanically induced neuronal activation.

With tips larger than 12-15 μm, there appeared to be significant leakage of chemical from the tip of the pressure injection electrode due to hydrostatic pressure (Fig. 7B). A vacuum line was installed to investigate the possibility of using back pressure to limit fluid leakage (Fig. 7A), and it was determined that vacuum could indeed inhibit leakage. It was decided that the medium diameter tips (3-8 μm, Fig. 7C) were adequate to accomplish the goals of the statement of work and further investigation of larger tip diameters was not necessary (see Investigation of Multiple Delivery Systems for further discussion).

Another significant problem was the effect of capillary action. As discussed below (Fig. 8), to maximize safety, minimize contamination and maximize commercial efficency, only small volumes were loaded into the tips of the chemical delivery pipettes. [Because of the extremely small volumes (picoliters) that are pressure injected from the pipette, fractions of microliters in total volume are adequate.] However, because the capillary pipettes are not completely filled, there is a negative capillary pressure (Fig. 7B) in aqueous solution that causes the stimulus fluid to migrate up the pipette and suck recording media into the stimulus pipette. This effect causes a significant dilution of stimulus chemical and, more importantly, loads the tip of the pipette with recording media. Hence, it is necessary to create a back pressure to compensate. Our fabrication consultant built a variable pressure gauge to regulate the back

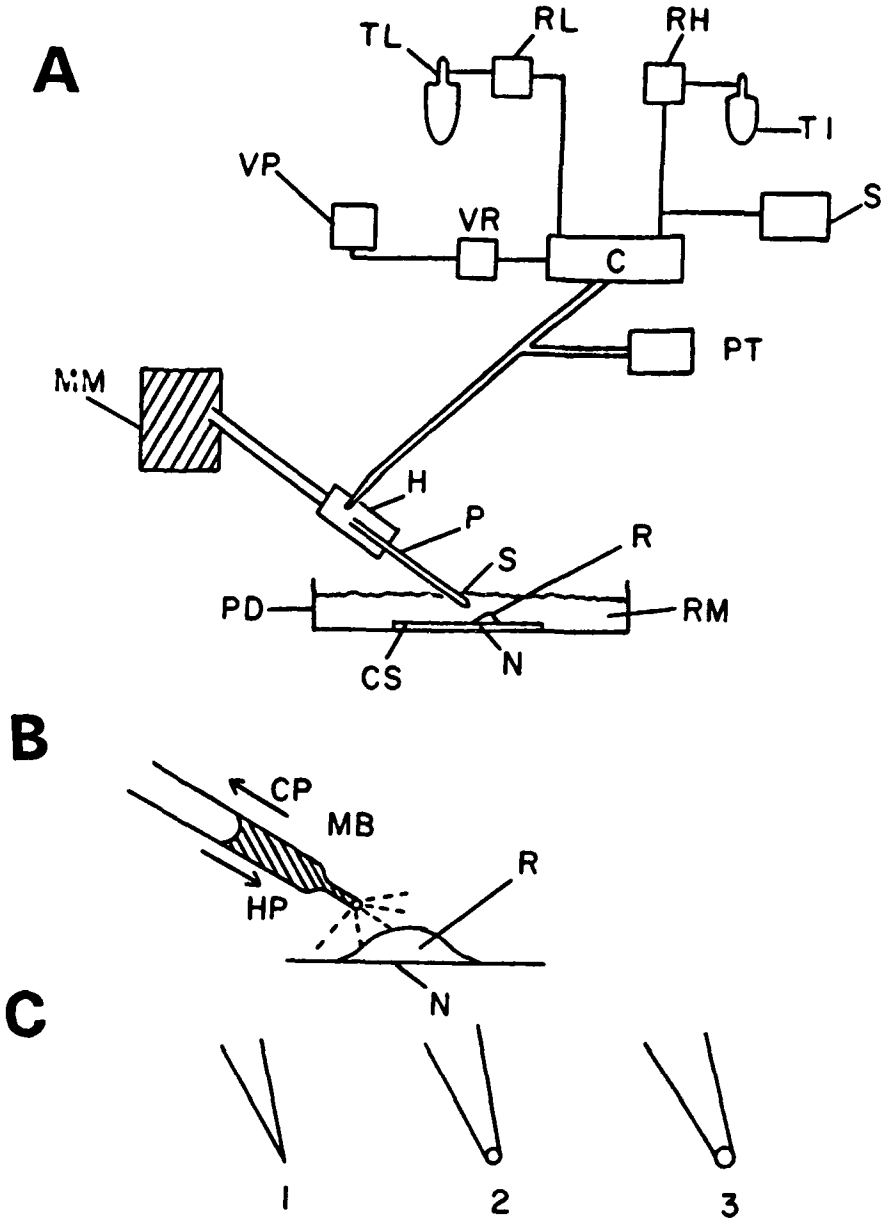


FIGURE 8. Safety procedure for loading and handling pipettes with toxic chemicals. The goal was to perform the pipette loading and disposal procedures under ventilation hood and to carry only a small volume (<1.0 μl) of solution to the experimental setup. A: shows a manipulator (M) being used to lower the micropipette (P) into the test chemical (TC). The pressure injection microelectrode is held in a microelectrode holder (EH) which is specifically designed for pressure injection, with an O-ring (O) to form an air-tight seal and a side port (SP)... (*figure legend continued on next page*)

FIGURE 8 continued. ...which is connected to a vacuum pump. The manipulator is clamped to the rod that is screwed into the holder. When the vacuum pump is turned on, a small (S) volume of the test chemical (TS) is front loaded into the tip of the pipette. Using the tip diameter of 2-3 μm or greater, it is possible to use this front loading procedure which is a much safer method for handling toxic materials than back filling with a hand-held needle and syringe as is used traditionally. At the end of the experiment, the residual test chemical is transferred to a safety container (SC) for disposal. All these operations are performed within the ventilation hood. B: After the pipette is filled, the holder is removed from the manipulator, carried to the experimental setup, and mounted on the micromanipulator (MM). The holder is connected to the pressure line that is connected to the pressure controller (PC) and to a pressure transducer (PT). The signal from the pressure transducer is sampled by the computer to determine the precise timing of pressure injection. Under microscopic observation, the pipette (P) tip is aimed at the cell body of a neuron before the cell is impaled with the recording electrode (R) in order to eliminate the possibility that mechanical vibration would dislodge the pipette from the cell. C: After the experiment, the holder containing the pipette is removed from the micromanipulator and carried back to the ventilation hood where the pipette is placed in the safety container (SC). D: The holder is then washed and flushed with a syringe (S) filled with soapy water, dried, and readied for reuse.

pressure necessary to inhibit the capillary filling (Fig. 7A). The influence of tip diameter on back pressure is currently being investigated.

The pressure injection protocol follows that established by our consultant, Dr. Alcayaga, for use in studying the response of chemosensitive nodose ganglion neurons which had been grown in co-culture with chemosensory (i.e., glomus) cells from the carotid body. In this protocol, a pressure injection wave 500 ms in duration was pulsed at a 0.5 Hz rate for 2-5 repetitions and the response of the neuron monitored. A pressure has been installed to monitor the timing of the pressure injection pulse (see Figs. 9-21 which illustrate the effects of pressure injection of test chemicals).

Pipette Loading and Safety Procedures. As illustrated in Figure 8, a chemical delivery system has been designed which minimizes exposure to toxic agents. Using pipettes with tip diameters >3 μm has an advantage in addition to those discussed elsewhere that it is possible to suck solutions into the pipette instead of back filling with a syringe attached to a long needle, as is done traditionally. Our consultant, Dr. Alcayaga, advised that using pipettes containing filaments was not advantageous for pressure injection because although the filament added in filling, it also produced an uneven flow of solution out of the tip during pressure injection.

The protocol (Fig. 8) is to pull a pipette, verify that it is proper diameter microscopically, insert the pressure injection pipette into a commercially available pipette loader with an air tight O-ring seal. The pipette plus holder is then moved to the hood which contains the solution of toxic chemical and is attached to a micromanipulator. The pipette is then lowered down into a microcentrifuge cup containing the stimulus solution, attach a tube from the electrode holder to a vacuum pump, and suck the stimulus solution into the tip of the pipette.

The pipette holder is then transported to the tissue culture recording setup, mounted to a manual micromanipulator, and attached to the pressure injection hardware (Fig. 7). The pipette is gradually lowered intothe solution under microscopic observation until it is pointed at the center of the microscopic field, about 60-100 μm from the bottom of the petri dish. The base of the microscope is then moved to position a neuron for study. The neuron is impaled with the recording microelectrode. Because the pressure injection pipette does not actually touch the surface of the cell, it is not necessary to use a micropositioner with remote control. Instead, a relatively inexpensive manipulator (see above) is adequate.

Multiple Chemical Delivery. As part of the general task of investigating delivery systems, the possibility of designing a multiple injection pipette was investigated. These experiments were completed by Dr. Tuckett in association with consultant, Dr. L. Monti, who is an expert on chemical delivery to olfactory and carotid body chemosensory neurons. One possibility was to pull a multibarrel glass micropipette. The critical issues here are: (1) obtaining a reproducible and uniform tip diameter, and (2) being able to efficiently attach the exit end of each barrel to a pressure line so that each barrel could be independently injected. It was found that the Sutter puller was not well suited for pulling a multibarrel pipette because the heating filament was open on top and hence did not provide uniform heating of each barrel. A Kopf puller was tested; however, the clamp that holds the ends of the glass capillaries did not apply a uniform, symmetrical pressure to all the individual pipette barrels and hence crushed the

glass. In contrast, a verticle Narashige puller was more promising because the clamp that holds the pipette ends is a screw mechanism that applies uniform pressure. After a padding material was inserted to uniformly distribute the pressure from the clamp, it was possible to pull multibarrel pipettes with a uniform tip diameter (500x). It was concluded that with proper modification, multibarrel pipettes could be pulled for micropressure injection. To connect the individual pipette barrels to the high pressure line, it was discovered that the ultrafine disposable pipette tips that are currently available for hand-held micropipettors fit into the capillary glass and can be quickly attached using superglue for an air-tight seal.

Alternatively, it was found that ultra fine disposable pipette tips used with micropipettors are uniformly manufactured with inside tip diameter of about 150 μm. Hence, if sufficient vaccuum can be applied to keep the fluid from migrating out of the pipette tip, it might be possible to glue several tips together, cut them so that they are precisely the same length, load them individually, and use them as a multiple delivery system.

As a third alternative, the possibility of using separately mounted individual pipettes was investigated. Our fabrication consultant (Mr. Evans) built a dual holder that would mount two delivery pipettes on one micromanipulator. The holder was capable of changing the height and angle of the individual pressure injection pipette independently. However, it was discovered that the angle and the distance between the two (fragile) pipette tips was extremely difficult to align. Hence, this alternative did not seem practical for commercial application.

Another alternative is to mount the individual pipettes on independent manipulator systems. Although this technique is feasible, it might be impractical because of the difficulty in repositioning the pressure injection electrodes while recording from the cell without creating enough vibration to injure the cell. Also, it would be physically difficult to position several micromanipulators around the 35 mm petri dish used for recording.

In summary, the two most practical methods of multiple delivery are likely to be a multibarrel approach either using a multibarrle electrode with a modified vertical puller, or by using ultrafine pipette tips. Because it was possible to accomplish the statement of work of this project without a multiple delivery system, we focused our efforts in other directions.

Data Collection

Voltage Clamp

A series of experiments were performed with our consultant, Dr. E. Lasater, on the feasibility of using voltage clamp techniques to record from the neurons in culture. Our objective was to determine whether it was technically feasible to obtain a high impedance seal between the cell and the suction electrode. Initially obtaining a good seal was difficult. Under Hoffman optics, the surface of the cell bodies appeared to be irregular with ridges and valleys. Several tip sizes were tested on the suction pipettes using different types of glass (Sutter, WPI), and various shapes of tips as modified by flame polishing the under a microforge. Although these modifications produced an improved high impedance

seal, there was still significant leakage current. Slightly (<5%) decreasing the osmolarity of the recording media caused the neuronal cell body to swell slightly. Under the Hoffman optics (200-400x), some areas of the cell body surface appeared to be more smooth in appearance and patching these areas produced a significant improvement in seal; and as a result, fast changes in voltage-current relationships could be monitored.

In conclusion, two important technical limitations of voltage clamp were tested: (1) whether a high impedance seal (gigaohm) could be obtained, and (2) whether the cell body could be adequately clamped. In some neural systems, the size and geometry, axonal or dendritic processes do not allow the whole cell to be voltage clamped. In such cases, since the voltage cannot be controlled, the currents cannot be accurately studied. These experiments suggest voltage clamp studies are technically feasible in the assay system.

Current Clamp

Initially the focus has been on intracellular recording because the technique produces a significantly higher yield and is technically less demanding than voltage clamp techniques. For example, an employee of Topical Testing with a B.A. degree was trained within a few weeks to record intracellularly. In contrast, to use voltage clamp will probably require a doctoral level employee. In addition, the voltage clamp technique is more tedious. In patch clamp, the electrode must be changed each time it touches a cell surface. Hence, a significant amount of time is required to change electrodes. [In contrast, a single intracellular electrode can often be used for several current-clamp recordings. Each time a new recording electrode is used, several minutes are required to position the electrode near the cell under high magnification. In addition, the manufacture of patch clamp electrodes is time consuming because in most preparations they must be polished in a microforge. In contrast, intracellular pipettes can be used immediately after fabrication.]

On the other hand, voltage clamp can be used to study membrane currents in detail and hence document ion channel mechanisms.

Response to Chemical Stimuli

The following figures illustrate the response of neurons in culture to chemical stimuli. The fundamental hypothesis to be tested during the contract period is whether co-cultured neurons are chemosensitive and will respond to endogenous and exogenous irritant and pair-producing stimuli. Hence, the cultures have been surveyed with a number of chemical species at high doses of chemicals.

Control Stimuli. Initially, neurons were tested with vehicles that are likely to be used in subsequent experiments. The neurons were found to be unresponsive to distilled water (Fig. 9), polyethylene glycol (Fig. 10), a nonionic hyperosmotic solution of 10% sucrose (Fig. 11), saline, and recording media.

Painful, Irritant and Toxic Stimuli. Neurons were found to be reactive to bradykinin (Fig. 12), capsaicin (Fig. 13: a classical pain-producing substance found in hot peppers), serotonin (Fig. 14), histamine (Fig. 15), and sodium cyanide (Fig. 16).

Acidic and Basic Solutions. Neurons were responsive to acetic (Fig. 17) and hydrochloric (Fig. 18) acid as well as to Hank's solution whose pH had been shifted to 4.9. At the other extreme, neurons were responsive to Hank's solution at pH 11.6 (Fig. 20) and sodium hydroxide (Fig. 21).

Results to date have shown that not all neurons that hav been recorded from are chemosensitive. This result is expected, since trigeminal neurons innervate a wide variety of tissues and supply neurons which are known to not be chemosensory, such as mechanoreceptive neurons. On the other hand, as shown in Figures 12-21, a substantial number of neurons recorded in culture have been chemosensitive and have reacted to a number of endogenous and exogenous substances known to produce pain and irritation as well as to shifts of pH that are known to be irritant.

Next year's research will focus on determining the classes of toxic compounds to which the neuronal assay is specifically reactive, whether response specificity is altered when the neurons are grown with corneal epithelium, and whether response specificity changes as the neurons are maintained for longer time periods in culture.

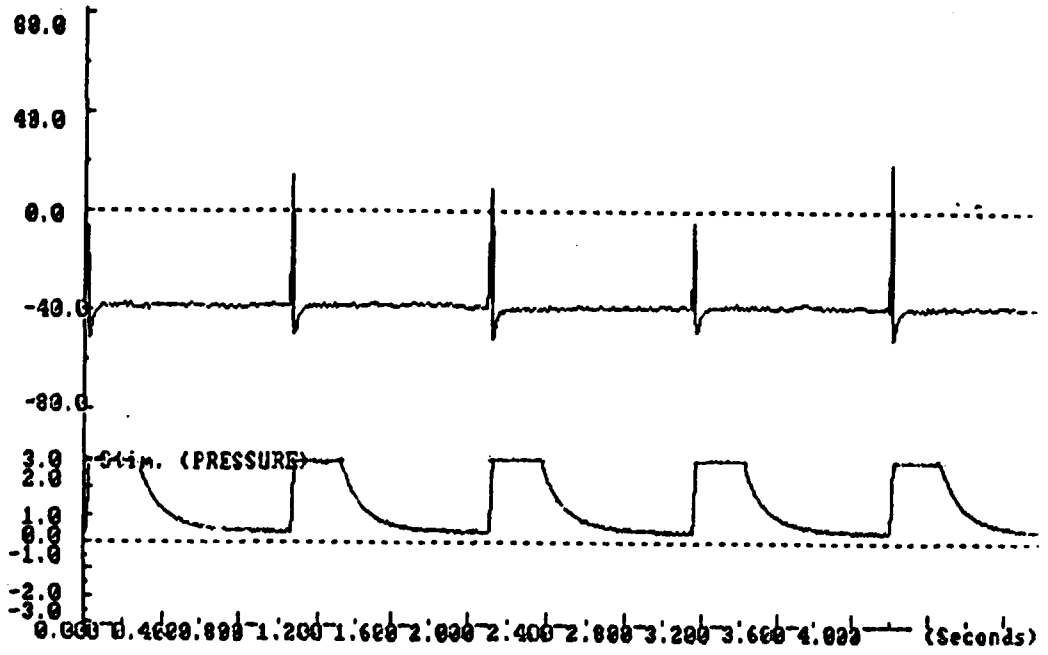


FIGURE 9. Stimulation with distilled water (control experiment). Plot shows a lack of response of the neuron during and after pressure injection of distilled water using the micropipette technology discussed above. The plot is typical of the plots in the following figures (Figs. 10-21). The top trace shows the membrane voltage and the left-hand axis (Y axis) is scaled in millivolts from +80 - -80 mv. The X axis is time extending from 0 to 5 sec. The bottom trace shows the timing of the pressure injection as monitored with a pressure transducer in the line (see Figs. 7 and 8 for details). In Figures 9-11 and in some other experiments (which will be explicitly designated), simultaneous with the start of each pressure wave (which usually lasted about 500 msec) a suprathreshold constant current stimulus pulse (50 ms) was generated which created a depolarization of the membrane potential. Hence, a membrane depolarization can be seen immediately before each pressure wave. This added stimulus was used to determine whether the chemical stimulus was producing a consistent change in the sodium channel activation of the neuron's membrane. The bottom trace is the pressure wave which is uncalibrated and merely shows the timing of each pressure stimulus. A series of 5 pressure pulses and electrical stimuli were given. There was no consistent change in the resting membrane potential or on its response to current stimulation. Hence, it was concluded that distilled water had no significant effect on the response of the neuron.

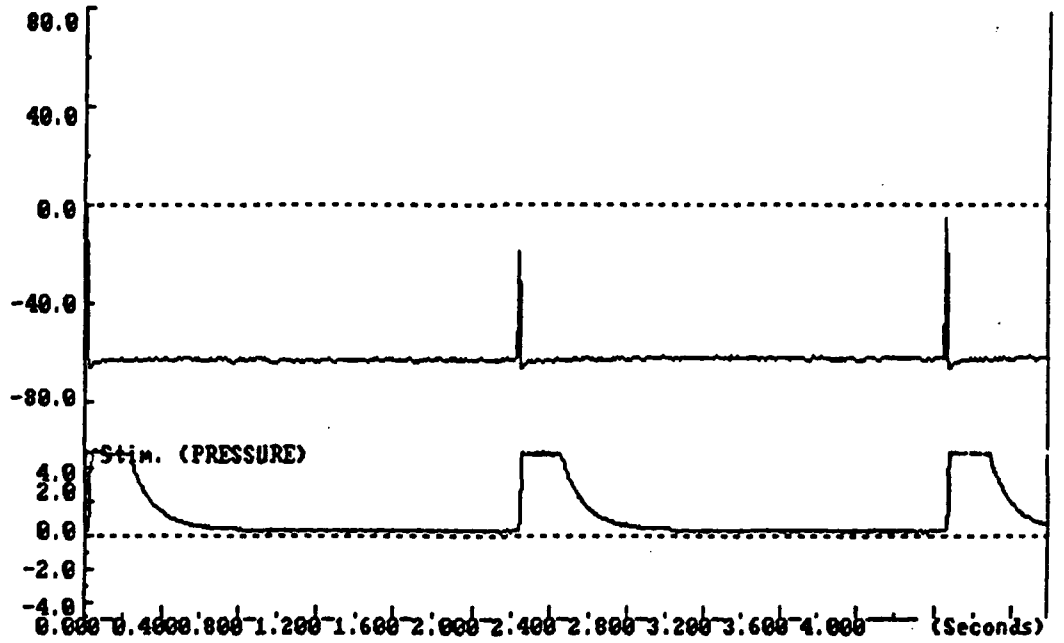


FIGURE 10. Stimulation with polyethylene glycol (control experiment). The stimulation procedure was as in Figure 9 with an identical time period of 5 sec of sampling during which three pressure waves and three electrical pulses were generated. Note that the polyethylene glycol had little or no effect on resting membrane potential or on sodium channel activation with the depolarizing electrical pulse.

FIGURE 11. Stimulation with 10% sucrose (control experiment). The stimulation procedure and figure scaling are as shown in Figure 9. Note that the pressure injection of the high molarity solution had little or no effect on the neuronal response. This result suggests that the presentation of a high molarity solution (in and of itself) has little influence on the neuron's response characteristics.

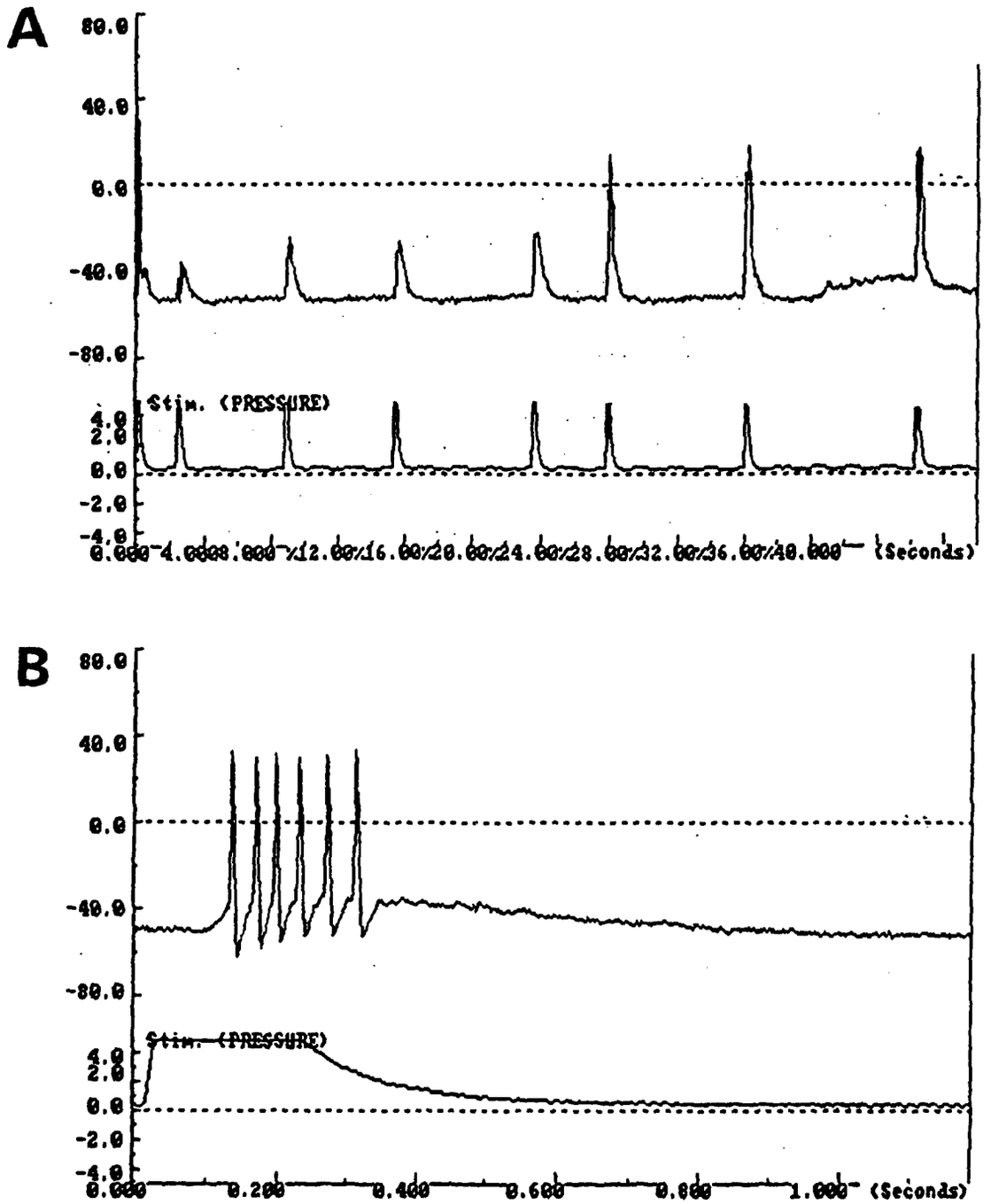


FIGURE 12. Stimulation with bradykinin (9.3 mM) in saline. A: Scaling is as in previous figures; however, the time course was for a longer period of 0-50 sec rather than 0-5 sec as in the previous figures. Note the continued responsiveness of the neuron to bradykinin. Due to the slow time base, the individual action potential firings are not seen in panel A. B: An expanded time scale of the stimulation of the portion of the recording in panel A from 0-1.2 sec. Note the multiple firings of the neuron following bradykinin administration (*figure legend is continued on next page*).

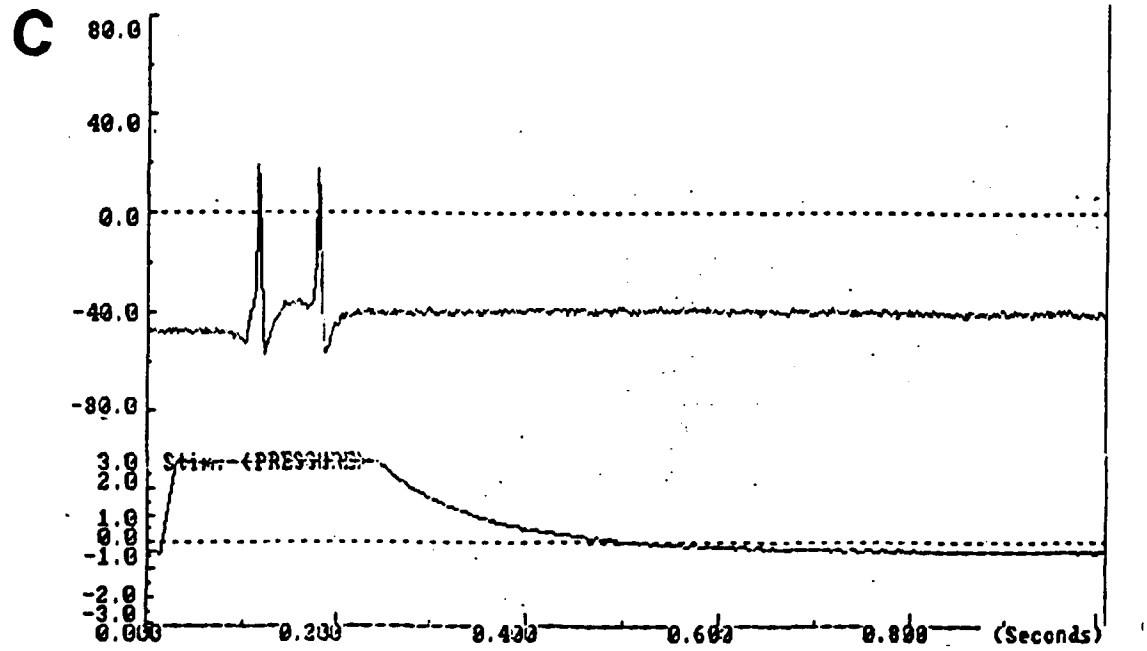


FIGURE 12 continued. C: shows the response of a neuron from a different culture to the same concentration of bradykinin.

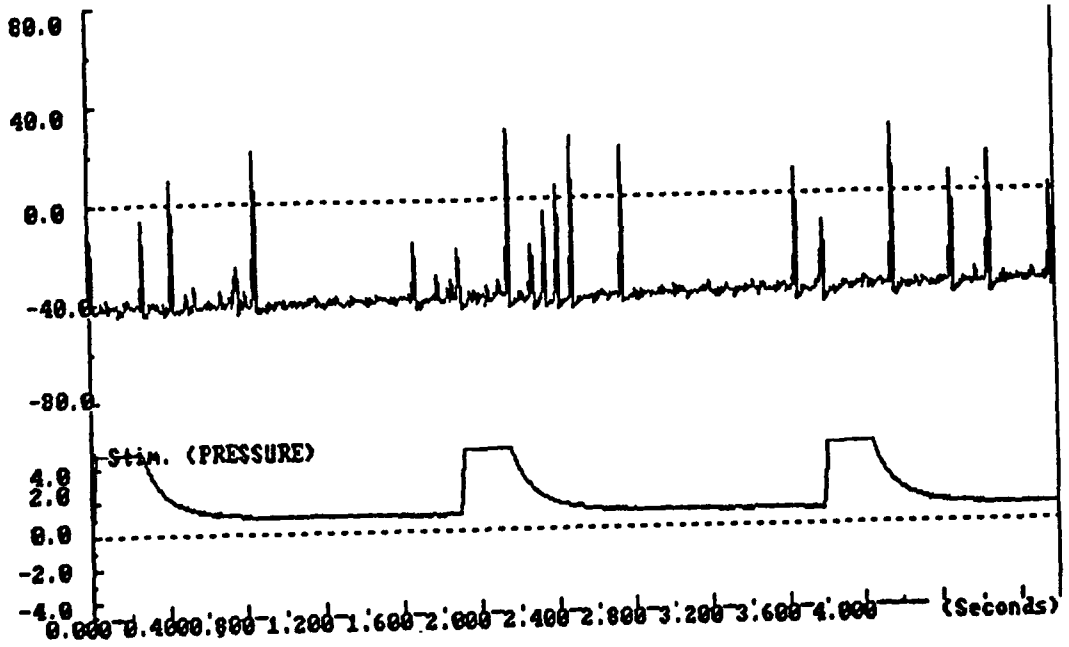


FIGURE 13. Stimulation with capsaicin. Stimulation is as in Figures 9-11 in that a constant current depolarization pulse was given at the first of the pressure wave and can be seen as the depolarization at the initial part of the pressure wave. It can be noted that there was no significant alteration in the sodium channel activation that can be seen with the electrical current. However, the capsaicin produced a significant reaction of the sensory neuron following the chemical injection.

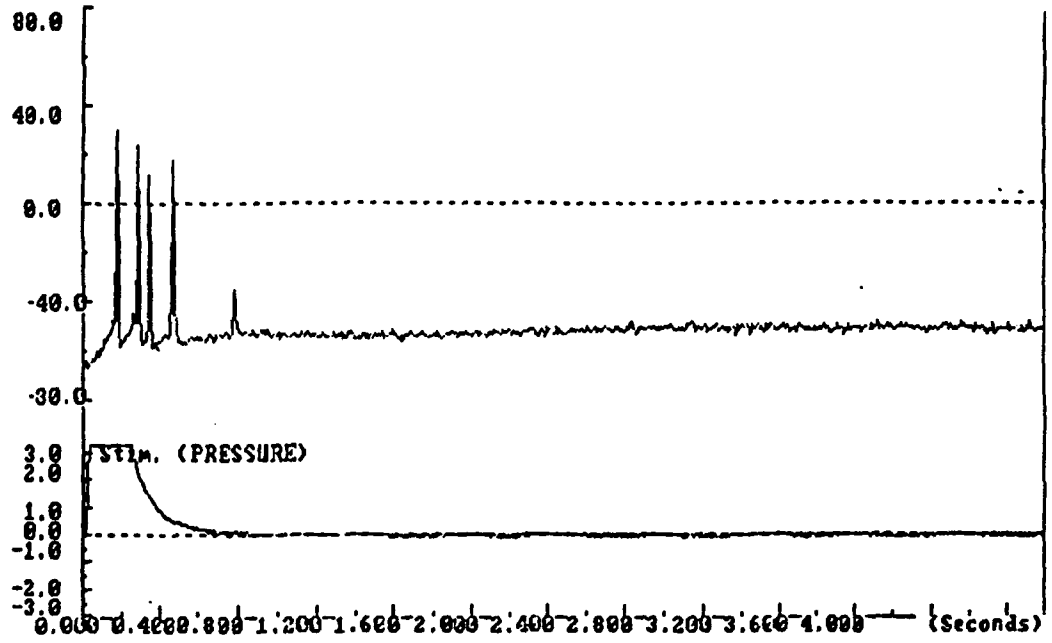


FIGURE 14. Stimulation with serotonin. Shows response of the a neuron in co-culture with corneal epithelium to serotonin (10.3 mM) dissolved in Earl's solution.

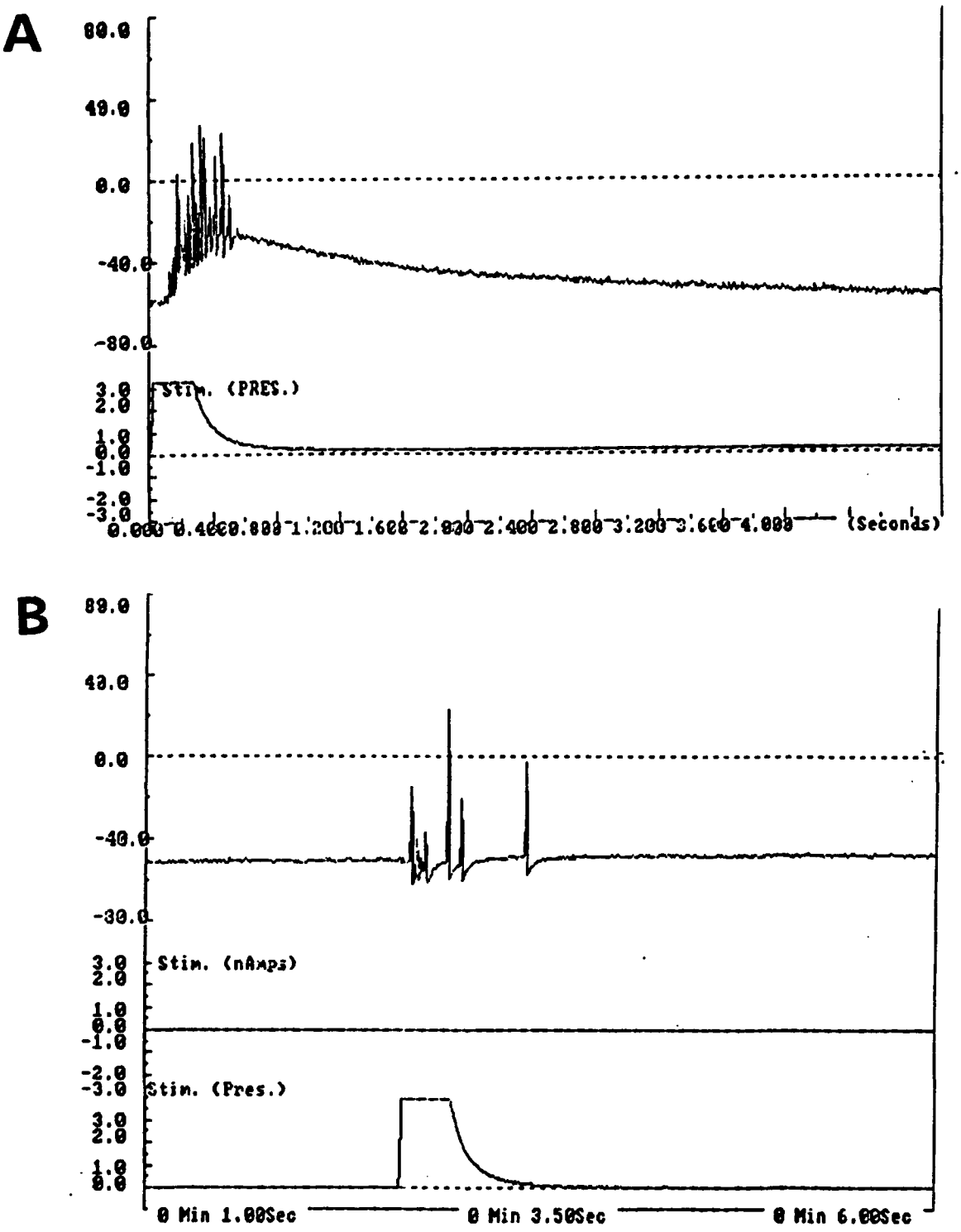


FIGURE 15. Stimulation with histamine (10 mM) in Earl's solution. A: The neuron responds vigorously to 10 mM solution, the response consisting both of action potential generation and a depolarization which lasts for several seconds. Total time is 0-5 sec. B: Response of another neuron to a similar dose of histamine (7 mM). Note that although there was action potential generation, there was no significant depolarization of the membrane potential on this run and no electrical stimulation was given.

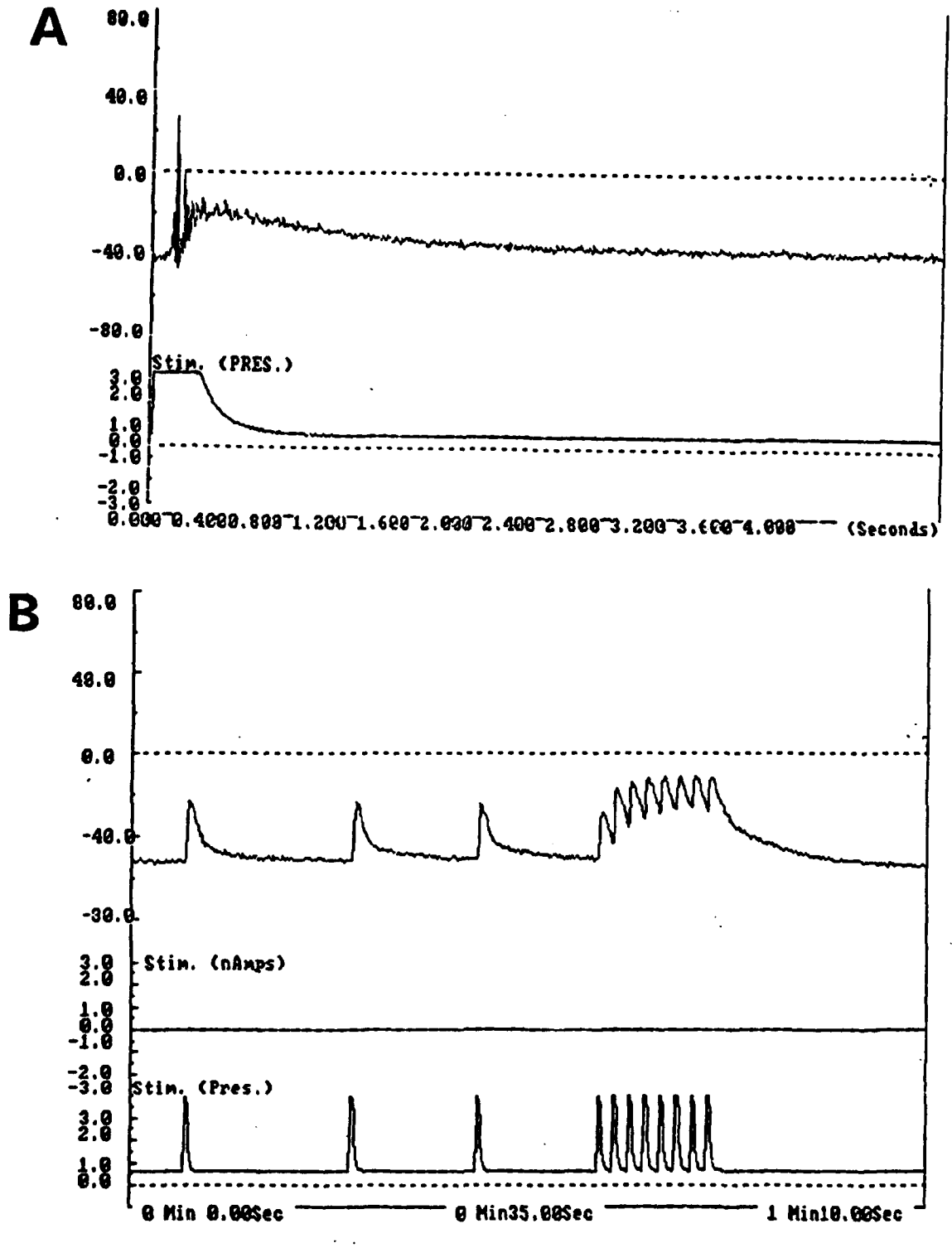


FIGURE 16. Stimulation with sodium cyanide (15 μg/liter). A: Note the firing of the nerve and the depolarization with recovery within the 5 sec time scale which does not return to the prestimulus resting membrane level within the 5 sec stimulus time. B: Shows the response of another neuron to the same dose of sodium cyanide over a compressed time scale of 1 min 10 sec. Note the depolarization of the membrane which can recur with repeated activation.

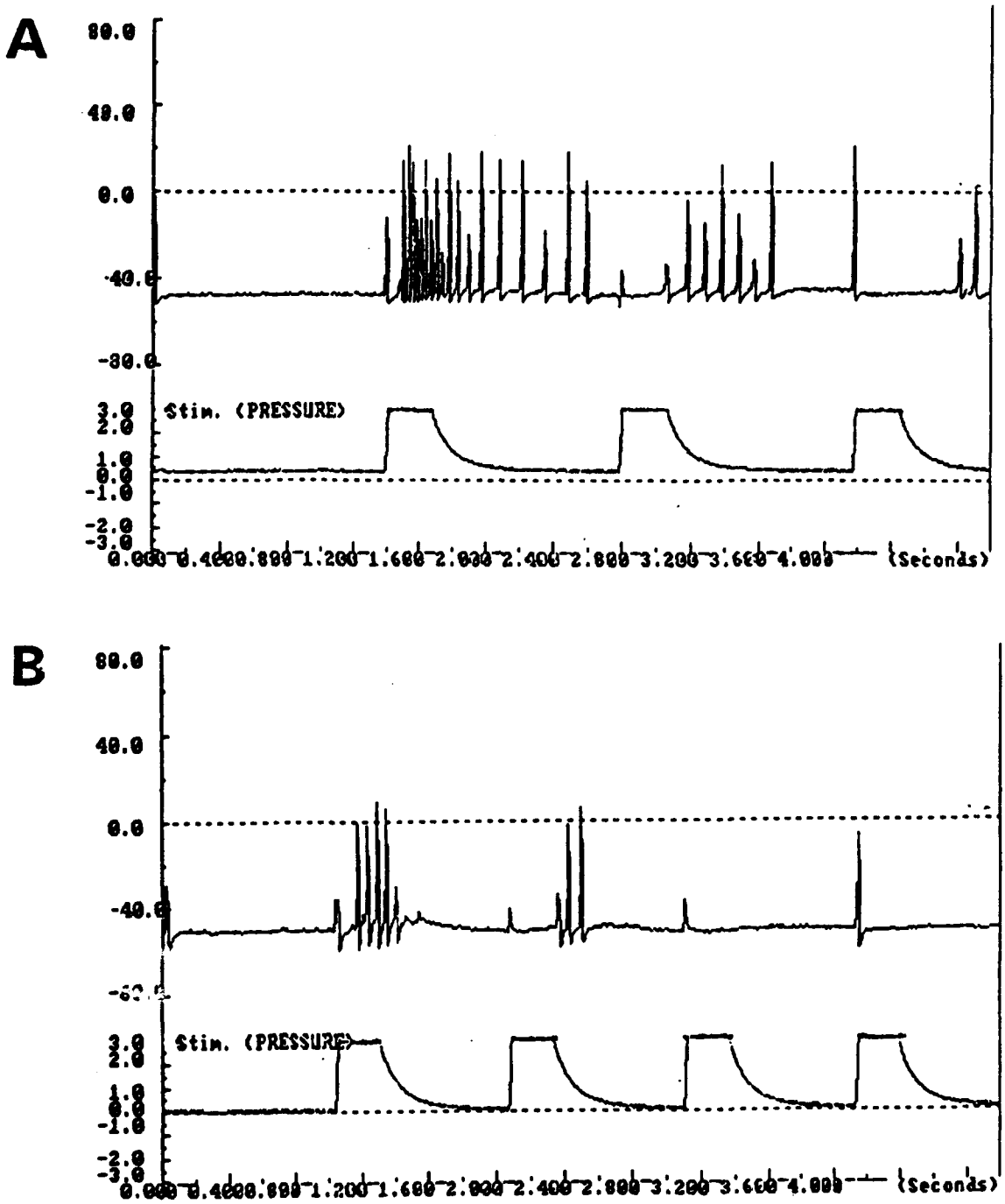


FIGURE 17. Acetic acid stimulation. Shows the response to increased concentrations. A: Response to 1 mM acetic acid. Note that as in Figures 9-12, a 50 ms constant current pulse was passed at the start of each pressure injection (as can be seen by the depolarization of membrane potential at the beginning of the pressure wave). The neuron was relatively sensitive to 1 mM acetic acid (repeated firing) but exhibited no significant membrane depolarization. B: Shows a response of another neuron from the same culture which was less sensitive than the previous unit to a higher dose of acetic acid (10 mM) (*figure legend continued on next page*).

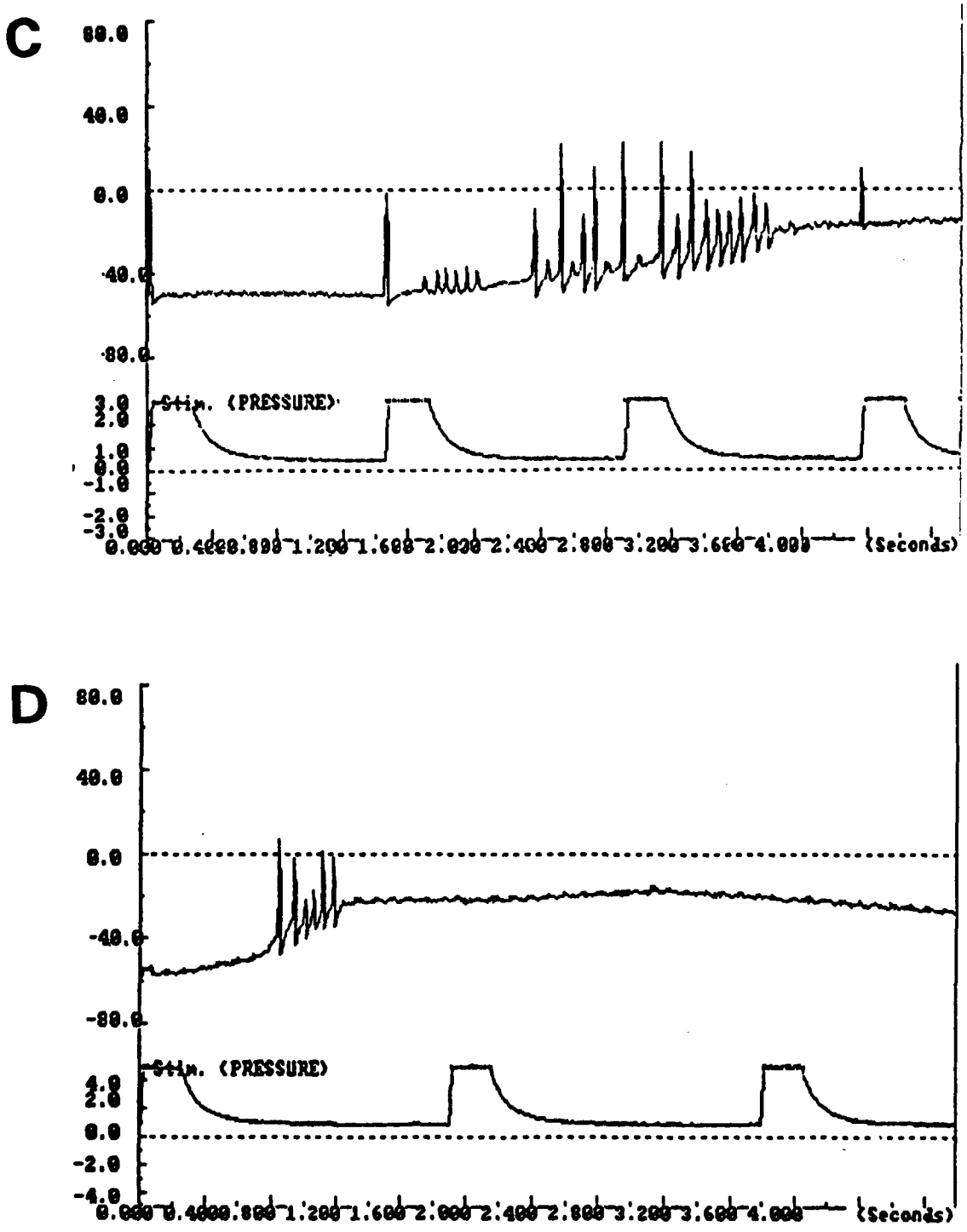


FIGURE 17 continued. C and D: Show the response of two different neurons to 100 mM acetic acid. In both instances there is not only firing of the neuron but also a significant depolarization. Hence, these initial data suggest that as the pH is lowered, acetic acid produces not only repeated firing of the neuron but also significant membrane depolarization.

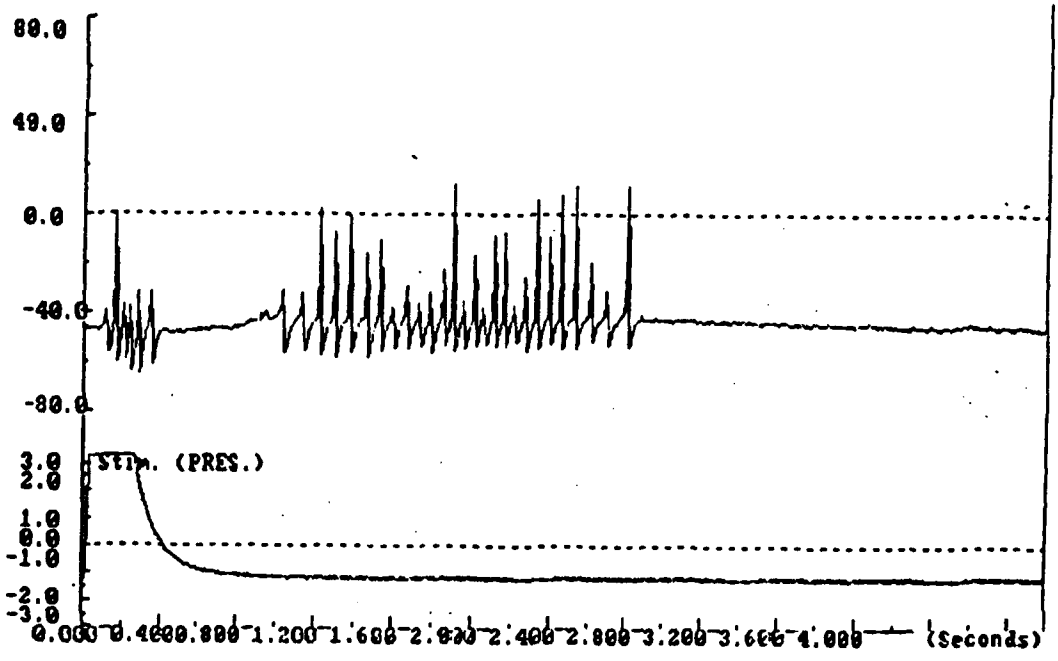


FIGURE 18. Stimulation with hydrochloric acid (1 mM). The record shows the sensitivity of this neuron to exposure to hydrochloric acid and repeated firing of the neuron following exposure. Note that the depolarization of the neuron was not significant.

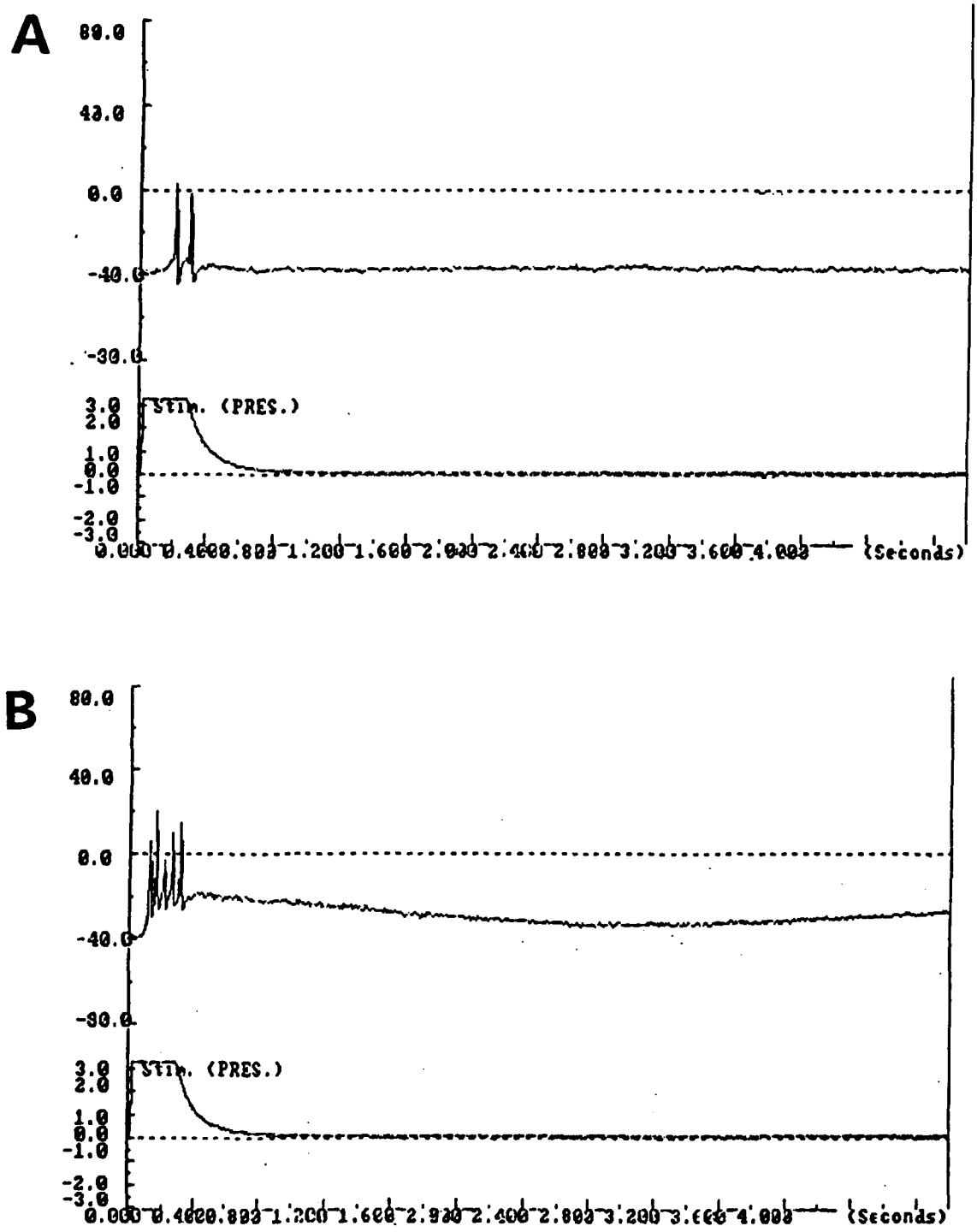


FIGURE 19. Stimulation with Hank's solution at pH 4.9. Panels A and B contrast the responsiveness to neurons from the same culture dish to the same stimulus. Note the difference in sensitivity of the two neurons to the same stimulus.

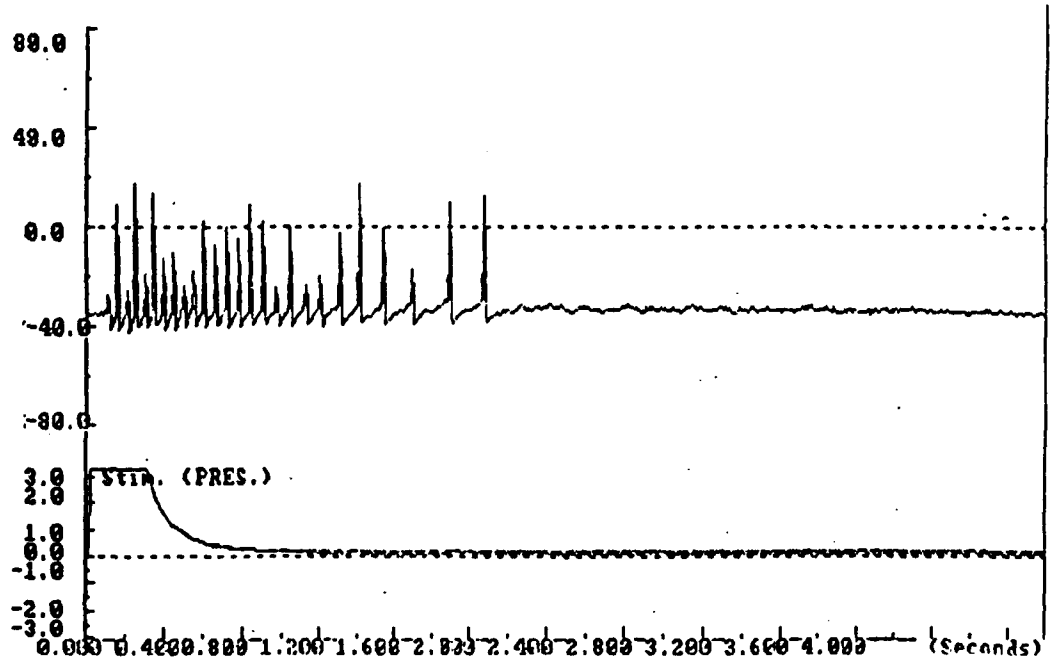


FIGURE 20. Stimulation with Hank's solution adjusted to pH 11.6. The neuron was reactive to recording media after being shifted to basic pH.

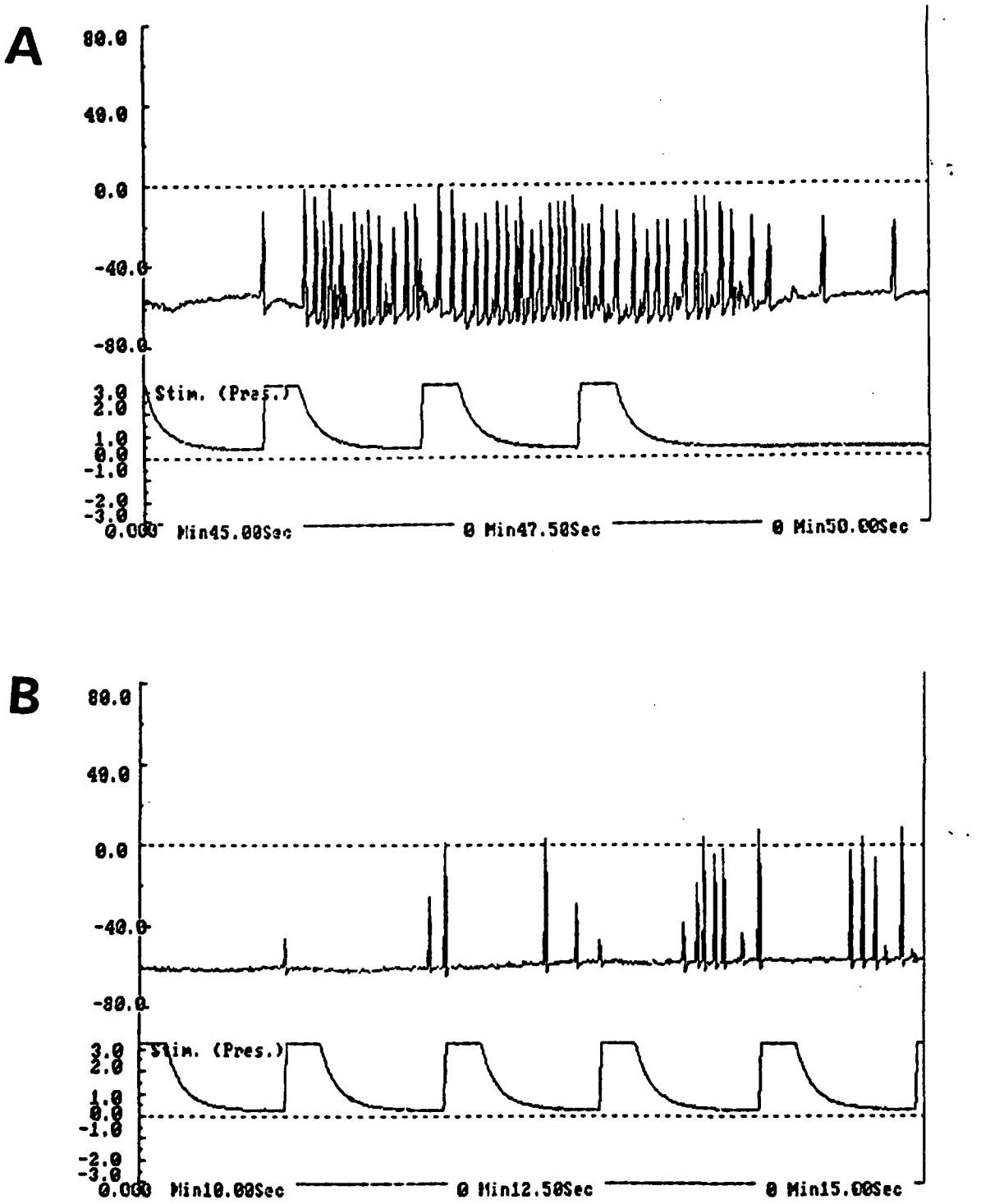


FIGURE 21. Stimulation with sodium hydroxide. A and B: show response of neuron to 1 mM sodium hydroxide in two different units. Note the greater sensitivity of neuron in A to the exposure to the basic solution (*figure legend continued on next page*).

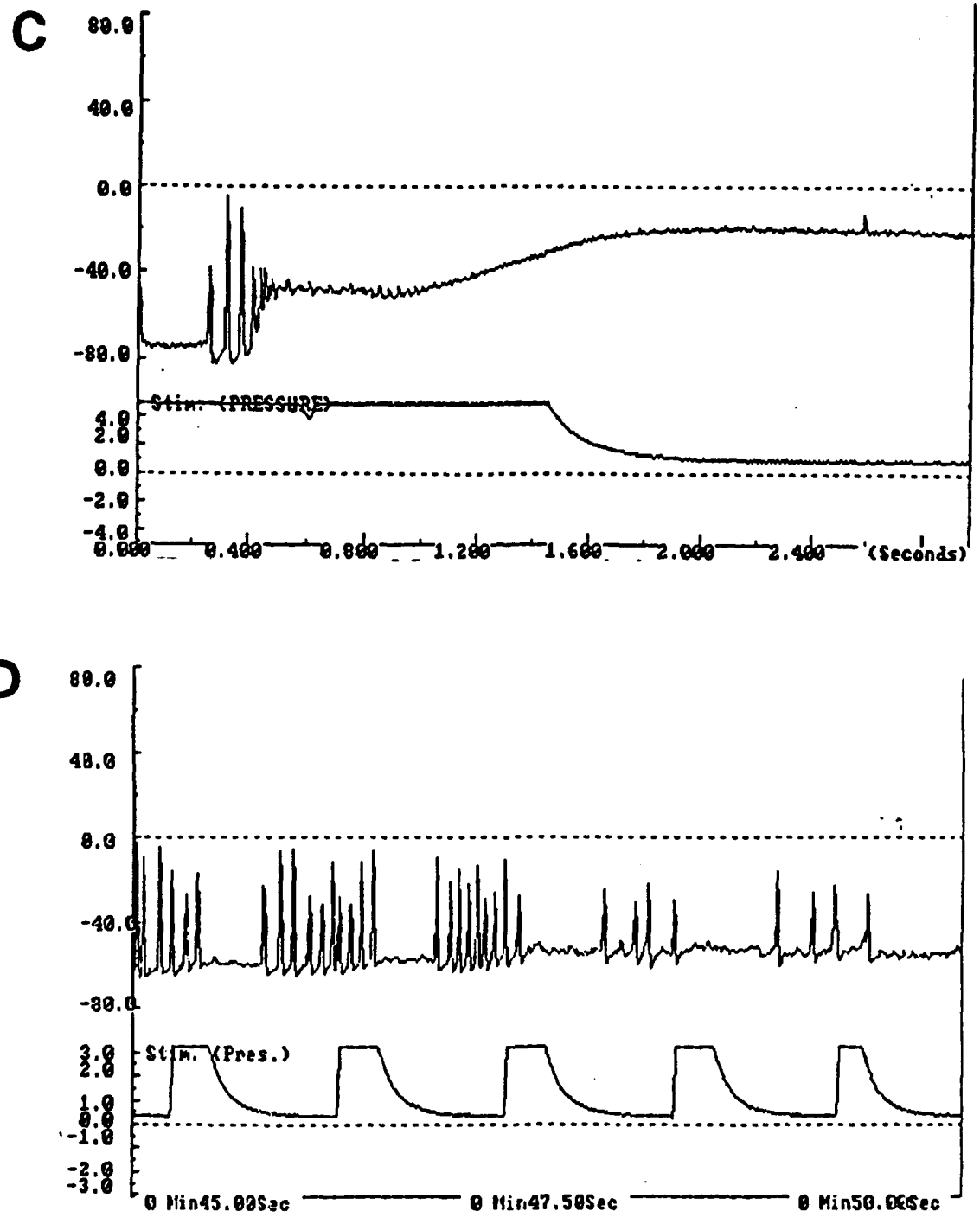


FIGURE 21 continued. C: Shows the response of a third unit to 10 mM sodium hydroxide. Note the accumulative depolarization of the membrane potential with each pressure injection as well as the fatigure of firing to repeated stimulation. D: Response of neuron to an extended pressure injection of one normal sodium hydroxide with a depolarization of the membrane potential following the firing.

PROGRESS TOWARDS ACHIEVING THE SPECIFIC OBJECTIVES

During the first year, substantial progress has been made toward establishing a working assay system. Focus has been on the technical problems in the experimental setup, on the complexities of establishing a consistent, reproducible co-culture, and on delivery of endogenous and/or irritant chemical stimuli. More specifically, emphasis has been on obtaining: (1) mechanical and (2) electrical stability, (3) digitization and (4) data collection, (5) chemical delivery, and (6) tissue culture reproducibility. The details of these efforts are described in the preceding section (see Progress to Date). The types of chemical stimuli used during this time period have focused on endogenous substances, most of which are known to produce pain and irritation as well as some general irritant stimuli such as acidic and basic solutions.

A summary of progress towards specific objectives is outlined below, as well as the tasks to be completed in the coming year. The GANTT chart in Table II above shows the originally anticipated work schedule. The first GANTT chart in the next subsection (Table II) summarizes the work completed to date. The anticipated work schedule for the coming year is given in Table III.

1. *Selection of a standard pollution stimulus.* Because of the variety of chemical species that have been applied to the rabbit eye in the Draize test, the experimental strategy of this contract is to apply chemicals from a variety of chemical families to determine the specificity of response and relate the specificity to previous in vivo test results. During the first year, a variety of chemical stimuli have been applied, including endogenous substances such as histamine and bradykinin, serotonin, etc., as well as extremes in pH and the classical pain-producing substance, capsaicin. During the next year, focus will be on the application of toxic chemical species to the in vitro system and document the differences in response characteristics.

2. *Develop a perfusion system which can rinse away the chemical stimulus in between stimuli.* As discussed above (see Progress to Date), the perfusion system has been implemented as well as a means for cleaning of the perfusion pathway.

3. *Evaluate how long cultures must grow before testing.* It has been found that the neurons require 2 1/2 to 3 days in culture before they begin to exhibit normal membrane characteristics. After 5-6 days, some neurons in culture begin to die. Initial observations suggest that there may be increased specialization in neuronal chemosensitivity of the cells that remain in culture. Hence, the working hypothesis is that specialization occurs as neurons continue to grow in culture for longer periods. The data gathered in the coming year should help in testing this hypothesis.

4. *Evaluate different data acquisition systems to determine which best suits current application.* Data acquisition systems and computer systems have been evaluated and a optimal system has been configured for our application. Hence, this task is essentially complete.

5. *Continue development of action potential recognition and data summary software.* At this point in time the most essential elements of the data acquisition software have been implemented. Up to three channels can be

simultaneously sampled at 10 KHz rate and the data can be directly stored on hard disk using a PC computer (20386, 33 MHz, with co-processor and hard disk with <20 ms random access time). Hence the neuron's response, electrical stimulus and pressure wave that signals the timing of chemical injection of chemicals can be simultaneously sampled. Consequently, the assay system is capable of recording as many minutes as necessary of the membrane potential and stimulus waveforms online and in real time. The gains in efficiency over storing on tape for later analysis are significant. The data is efficiently packed, and a backup system which uses cassette tapes (120 Mbyte) is working efficiently. Software for automated action potential recognition (and frequency of neural firing) after chemical injection is currently being implemented. It is anticipated that the software systems will continually evolve and improve during the coming year. Specifically, target areas include: (1) the completion of the automatic action potential recognition and interspike interval calculation software, (2) improve techniques for obtaining publication-ready data plots on the laser printer, (3) more efficient operation of software for calculating membrane properties, and (4) data base management strategies for organizing and statistically testing the populations of experimental data. Hence, task #5 will continue throughout the contract period but at a lower level than during the first year.

6. *Make improvements in the total integrated system.* The total assay system is complex, and many changes have been made that have improved system performance. Focus has been on design of the chemical delivery system, and a preliminary analysis has been made of the feasibility of a multidelivery system (see Progress to Date). Although minor changes will continue to be made, the level of effort in this area will be significantly reduced during the coming year.

7. *Evaluate current clamp, voltage clamp and fluorescent technologies.* The current clamp technique is the standard assay used for analyzing membrane properties of the neurons in culture. As explained above, constant current can produce reliable data with with which to reach the goals of this contract. During the first year, a feasibility study on the use of voltage clamp proved to be favorable. The data suggests that it will be to obtain patch-quality recordings on the surface of the trigeminal neurons in culture and that the voltage clamp electronics will be able to clamp the neuronal processes in culture so that accurate measurements of membrane current will be possible (see Progress to Date). These data also suggest that using patch clamp technology to obtain recordings from single membrane channels will also be feasible. Hence, the proposed evaluation of voltage clamp recording is essentially complete. An investigation of fluorescent technologies will be performed during the coming year.

8. *Determine whether cell inhibitor(s) significantly influence the qualitative response to the chemical stimulus in co-culture.* This task is generally related to the issue of isolation of the neurons from fibroblasts so that a more pure co-culture of neurons and epithelium can be obtained. There are three strategies which are being investigated to limit fibroblast proliferation: [1] As described above (see Progress to Date), it has been discovered that the keratinocyte growth medium has a significant inhibitory affect on fibroblast proliferation, while at the same time promoting epithelial proliferation. [2] It has been found that the neurons can be separated from the fibroblasts by preplating on plastic

jto which the fibroblasts adhere quickly and then replating the neurons (i.e., a differential adhesion technique). [3] It may be necessary to add a cell inhibitor for a short period of time. For example, evidence now suggests that neurons added an established epithelial culture might not establish a firm contact with substrate and hence may be more difficult to impale and record from. Hence, the temporary addition of a cell inhibitor to the neuronal culture may be necessary to limit fibroblast and/or epithelial proliferation while neurons are establishing contact with the collagen substrate. It is anticipated that this task will be completed within the first six months of the coming year.

9. *Determine whether cell density qualitatively affects chemical stimulus response.* This task has not yet begun but will be completed during the second year (see Table III).

10. *Determine whether the buffer system significantly influences the qualitative response to chemical stimulus.* Comparison of neurons recorded from HEPES and bicarbonate buffer systems has shown no significant differences in response to date. More experiments will focus on this issue in the coming year.

11. *Determine whether perfusion solution temperature qualitatively influences response to the chemical stimulus.* To date, the experiment have been conducted at room temperature. Influence of temperature on neuronal response characteristics will be tested in the second year of the contract.

12. *Determine whether rabbit neurons have a qualitatively similar response as rat neurons.* This objective will be reached during the second year of the contract.

TABLE II

PROGRESS TOWARDS MEETING SPECIFIC OBJECTIVES

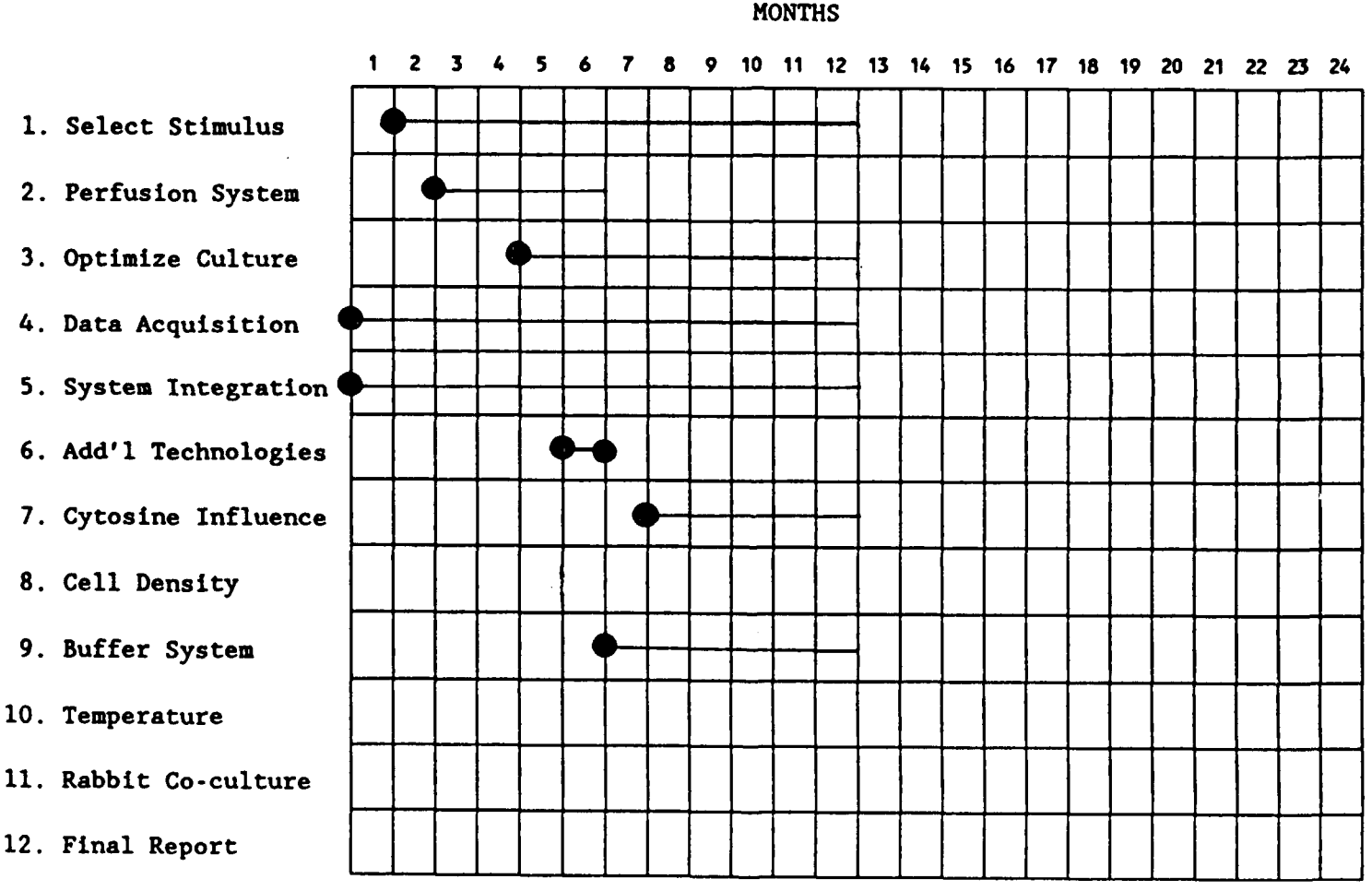
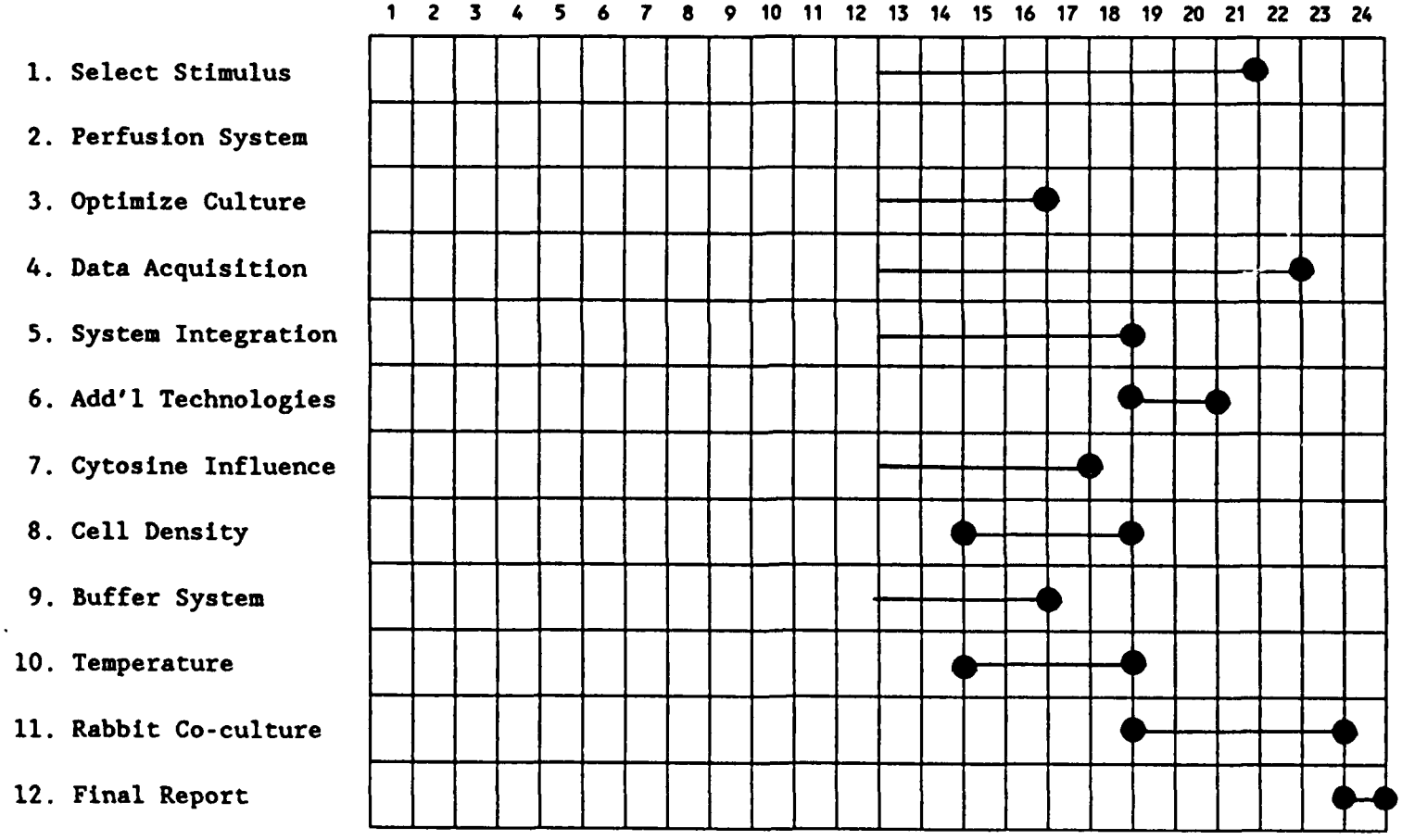


TABLE III

SPECIFIC OBJECTIVES FOR SECOND YEAR

MONTHS



PUBLICATIONS

A manuscript is being planned which will be submitted to an appropriate toxicology journal describing the physiological response of our assay system. We plan to test with examples from a number of specific categories of toxic chemicals and hence obtain data on response specificity.

PROFESSIONAL PERSONNEL

Topical Testing Personnel

Dr. Robert P. Tuckett	Director of Research
Mr. John H. Fisher	Electronics Specialist
Mr. Scott Murphy, B.S.	Computer Specialist
Ms. Susan Roberts	Senior Technician/Tissue Culture Specialist
Mr. Shawn Carlyle, B.A.	Junior Technician worked recording from tissue culture during development stage

Employees Through Subcontract At University of Utah

Dr. Julio Alcayaga	Research Associate, worked through subcontract with University of Utah in 1991. He helped with the design of the experimental setup and initial strategies of neural recording and chemical delivery
Dr. H. Martin	Research Associate, is working through University of Utah subcontract in 1991. He has provided expertise on response of nociceptive neurons to chemical stimuli
Dr. Jaime Eugenin	Research Associate, working through subcontract with University of Utah. He is currently evaluating response of neurons to irritant and toxic chemicals

Consultants	Expertise
Mr. Barry Evans	Fabrication and design
Dr. John L. Walker	Ionic solutions and membrane channels
Dr. Eric M. Lasater	Voltage clamp
Dr. Luis Monti-Bloch	Chemical delivery
Dr. Dana K. Vaughan	Histology
Dr. Kathleen B. English	Histology and culturing epithelium

INTERACTIONS

Conferences and Presentations

University of Utah Eye Institute, Clinical Conference, May 10, 1991. Presentation title: "Response of Trigeminal Neurons to Irritant Stimuli"

Interactions with Air Force Laboratories

Dr. Tuckett met with Dr. Channel and Mr. Del Raso on June 24, 1991. The experimental protocol and strategy were reviewed, and demonstrations were conducted in the laboratory and tissue culture areas in the subcontracted space at the University of Utah, Department of Physiology.

A trip to Wright Patterson is planned for the near future (February or March, 1992).

PATENTS AND INVENTIONS

A patent on the technology described herein was applied for last spring and DD Form 882 was submitted to our ACO on November 14, 1991.