MODEL P-80/PC

FLAMING/BROWN MICROPIPETTE PULLER

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INTRODUCTION

The Model P-80/PC Brown-Flaming Micropipette Puller combines a proven pulling technology with programmability to produce a very versatile instrument. The pulling mechanism is derived from the P-77/P-80 series of pullers, which have demonstrated the ability to pull a complete range of pipette profiles. Added to this mechanism is the ability to program different pulling sequences; thus, allowing ease of use for pulling a multiplicity of pipettes on one device.

The P-80/PC is a 'velocity sensing' puller. This feature allows the puller to indirectly sense the viscosity of the glass, giving the P-80/PC the ability to pull pipettes from all glasses save quartz. Even difficult to pull formulations, such as aluminasilicate glasses, are handled with relative ease.

The P-80/PC can fabricate pipettes for use in such researches as intracellular recording, patch-clamping, microinjection and microperfusion. However, realizing the full potential of this instrument is dependent on a complete understanding of the way it implements the pulling process. To this end we urge that this manual be read in its entirety. To aid in understanding the function of the instrument, sample programs are already loaded in memory (as discussed in subsequent material).

For that setting in which a number of users must work with one device, or for that single user whose investigations require a variety of pipettes; the P-80/PC is the answer.

SUTTER INSTRUMENT COMPANY

SECTION 1

UNPACKING AND SETTING UP

The Model P-80/PC is shipped to you wrapped in a plastic bubble wrap and surrounded by approximately 6 inches of loose fill on all sides. Please take note of this method of packaging. Should it ever be necessary to ship the puller to another location, the same method of packaging should be employed.

IMPORTANT: Improper packaging is a form of abuse, and as such can be responsible for voiding the warranty where shipping damage is sustained as a result of such packing.

All material shipped with the instrument is contained within or is attached to the plastic wrapping.

After removing the puller from its shipping carton, cut the tape holding the plastic wraps. Be careful not to cut through the power cord at the back of the device. Open the plastic cover by lifting the hinged front and remove any filament containers. These containers are usually located on the left side in front of the main valve on the nitrogen tank. Those pullers ordered without a nitrogen tank will have their filaments shipped in a small container attached to the front of the plastic wrap. While the puller cover is still raised, open the valve atop the nitrogen cylinder by turning it counter-clockwise (as viewed from the valve end of the cylinder).

If you have ordered your puller without a nitrogen cylinder, regulator and cradles it will be necessary to secure a separate cylinder and regulator to deliver nitrogen at 50 psi, via the attached length of tubing, to the gas solenoid. This nitrogen source need not be dry or medical grade; however it should not be a source that is subject to changes in moisture content (as is the case with most piped in sources). Should it be more readily obtainable, compressed air is also suitable, subject to the same moisture considerations.

Place the puller in a location where there is a free flow of fresh air on all sides. The fan draws air in through the vents on the sides and exhausts out both ends of the heat sink. **NEVER ALLOW THE FREE FLOW OF AIR TO BE RESTRICTED.**

Since the P-80/PC is a microprocessor controlled device, it should be accorded the same system wiring precautions as any 'computer type' system. If microprocessor based systems in the lab require line surge protection for proper operation, then the same protection should be provided for the P-80/PC.

FIRST TIME USE

Plug the instrument into a power receptacle of the correct voltage and frequency. NOTE: A power source that is essentially electrically noise free is desirable. The control circuitry of the puller uses digital logic that may be susceptible to transient spikes that can be caused by faulty wiring or noise producing machinery, such as centrifuges or other equipment utilizing SCR control circuitry on the same power lines.

Lift the front of the plastic cover; open the main tank valve located at the left end of the nitrogen tank. Open the valve fully (counter-clockwise). It should not be necessary to turn off this valve between electrode pulling sessions. The high pressure meter should read above 1500 psi. Adjust the regulator to a reading of 50 pounds on the low pressure meter. If this reading is at a value higher than 50 pounds, turn the regulator knob counter-clockwise one turn. No change will be observed in this pressure reading until an electrode pipette is pulled. The pressure will then drop. After several electrodes have been pulled, the pressure will stabilize at a lower value. Now readjust the pressure to 50 pounds.

Pressures greater than 50 pounds are not recommended.

Programs 0 and 1 are preprogrammed into the puller by the manufacturer. Both programs were developed using 1mm O.D. borosilicate glass with an I.D. of .5mm. Program 0 will pull a micropipette of less than .1 micron and program 1 will pull a patch-pipette of about 1 micron.

To change from one program to another press the reset button on the front panel, and then select the program on the keypad. DO NOT USE OTHER PROGRAMS OR CHANGE THE VALUES IN THESE PROGRAMS UNTIL YOU HAVE READ SECTIONS II, III AND IV OF THIS MANUAL.

To execute program 0 or 1, place a piece of glass in the carriers and press the pull key on the keypad.

SECTION 2

MECHANICAL DESCRIPTION, CONTROLS AND ADJUSTMENTS

This section presents a basic mechanical description of the P-80/PC, with particular emphasis on terminology. Knowing the names of the various parts greatly facilitates communication between the investigators and the factory when discussing adjustments or service problems. In addition, various controls and adjustments on the top of the instrument are located and described. Those adjustments which are considered part of maintenance procedures are dealt with in Section 6.

GAS SUPPLY TANK AND REGULATOR: The Model P-80 gas supply consists of a tank filled with nitrogen of approximately 2 1/2 pounds at a pressure of 1800 psi, a regulator with high pressure and low pressure gages, an electronically controlled solenoid valve, a micrometer flow valve, and a nozzle.

The gas should last several years with normal use. Continuous lowering of high pressure might indicate a leaky system, depending on usage. The tank may be refilled by any supplier of compressed gases. Refill only with nitrogen.

When installing a refilled tank, make certain all the fittings are tight.

CAUTION: Be certain to shut off the main tank valve before removing the tank or the regulator from the tank. The tank is at high pressure and should be handled with care.
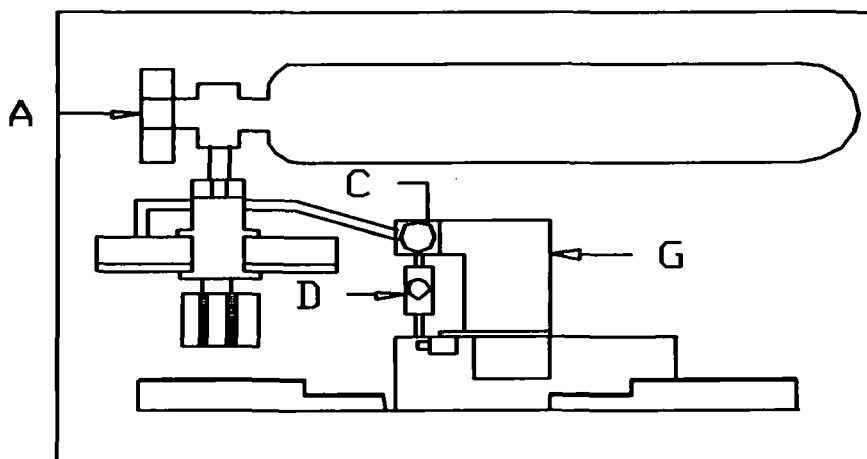

FIGURE 1

CYLINDER MAIN VALVE: (Fig.1, a) This valve is closed at the time of shipment. Upon receipt of the instrument, this valve should be opened all the way. This is a double seat valve and should not be left partially opened; small leaks may occur. If the instrument is to be left unused or stored for any length of time (two weeks or more) this valve should be closed.

NITROGEN REGULATOR: This regulator is fitted with an industry standard CGA 580 connection. The high pressure and low pressure gages read, respectively, cylinder pressure and

pressure of gas delivered to the micrometer flow valve. The large central knob adjusts the delivery pressure and is normally set at 50psi.

GAS SOLENOID: (Fig. 1, c) This solenoid is activated during the hard pull phase of the pulling cycle. It admits nitrogen to the micrometer flow valve.

MICROMETER FLOW VALVE: (Fig. 1, d) This micrometer valve (needle valve) controls the flow of gas to the filament. Despite the indicated range of values, this valve has a working range of approximately 100 units from no flow to maximum flow. The micrometer markings refer to thousandths of an inch and are referred to as numerical indication x 1000 (i.e. a reading of .1 inches is called 100).
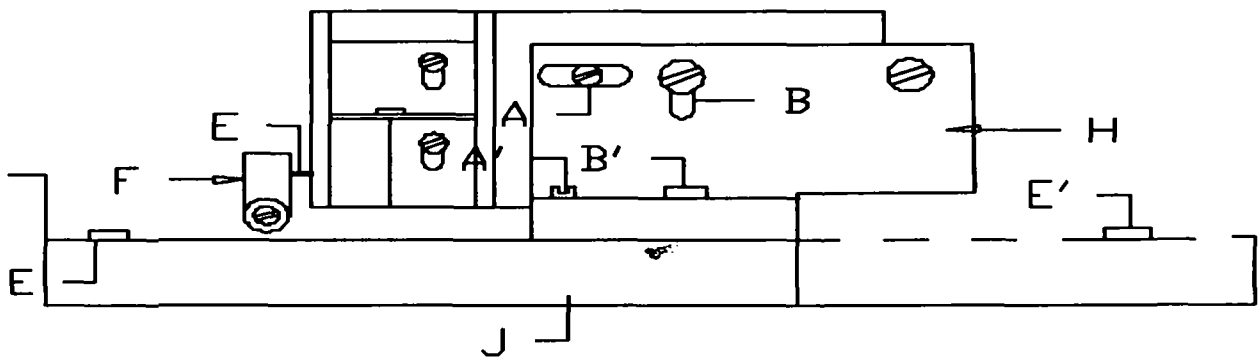

FIGURE 2

NOZZLE: (Fig. 2, e) The nozzle conducts gas from the flow valve to the filament area. It is a press fit into an o-ring located at the front of the flow valve. The nozzle tip is usually located from 1 to 2 millimeters below the filament and centered on it.

NOZZLE HOLDER: (Fig. 2, f) Maintains the nozzle in position below the filament. The holder contains two adjustments. The screw on the top allows the nozzle to be rotated and moved in and out of the front of the flow valve. The screw that secures the holder to the filament block can be loosened allowing the nozzle to move up and down.

FILAMENT BLOCK ASSEMBLY: (Fig. 1, g) The filament block assembly is made up of several pieces of hard black nylon. Wires supplying current to the filament are attached to threaded 'posts'. This current is carried to the filament via the upper and lower heater jaws. Note that these jaws are slotted and may be moved up and down by loosening the screws that secure them to the front of the filament block assembly. If the jaws are moved, make sure that the securing screws have been tightened; otherwise poor current flow can result. It is desirable to keep the gas solenoid/flow valve assembly lined up with the nozzle to prevent leaks at the o-ring. Note the two screws in slots at the back of the filament block assembly. Loosening these screws allows the gas solenoid/flow valve assembly to slide left or right.

ANGLE PLATE: (fig. 2, h) The angle plate secures the filament block assembly to the cover plate; it contains two important adjustments. Note the chrome plated screws in slots at points A and A' and the locking screws in slots at points B and B'. The chrome plated screws are 'eccentrics'; by rotating them with a screwdriver the filament block assembly can be moved up and down (A) or forward and back (A') to adjust the position of the filament. Loosen the locking screw associated with each 'eccentric screw' before turning, and tighten after completing the adjustment.

COVER PLATE: (Fig. 2, j) The cover plate conceals the entry of the pulling cables in to the base of the instrument. It is attached to the top by two screws, in slots, at points E and E'. Loosening these screws allows the filament block/angle plate assembly to move forward and back over large distances.

NOTE: The movements of the cover plate and the jaws constitute the 'coarse adjustments' of filament position, while the eccentric screws are the 'fine movements'.
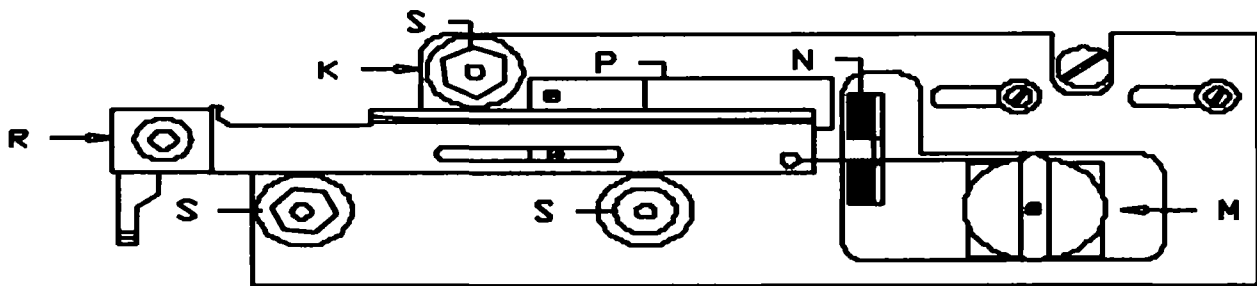

FIGURE 3

PANELS, LEFT and RIGHT: (Fig. 3, k) The panels are the angled surfaces that provide mountings for the puller bars and their bearings, the spring stops, the bumpers, and the upper cable pulley assemblies. Except for minor differences in shape, the left and right panels are identical. Note the three socket-head cap screws that attach each panel to the top. These screws are used to align the pullers bars. Their use, if necessary, is covered in the maintenance section.

UPPER CABLE PULLEY ASSEMBLY: (Fig. 3, m) This assembly conducts the pulling cables from the puller bars to the centrally located (and concealed) lower pulley assembly. Note that this assembly is attached to its panel by two screws, in slots, and contains a large eccentric adjustment screw (G). This eccentric screw is used to adjust cable 'tension' its use is covered in the maintenance section.

BUMPERS: (Fig. 3, n) The bumper stops the motion of its associated puller bar.

SPRING STOP: (Fig. 3, r) This assembly consists of the puller bar, glass clamp, clamp (wing) nut and cable retaining screw. The puller bar is made of mild steel and coated with a controlled thickness of hard chrome. Glass is loaded into the groove near the tip of the puller bar and is held in position by tightening down the clamp nut. The cable retaining screw holds the cable in a shallow groove at the end of the puller bar, and forms the 'resistance' against which the cable ends pull.

V-BEARINGS: (Fig. 3, s) These bearings are the guides for puller bar motion. They are made of stainless steel and should never be oiled (see maintenance section). Note that these bearings are mounted on stainless steel bushings one of which is round with the other two being hexagonal. The hexagonal bushings are used to adjust position and ease of travel of the puller bars (see maintenance section).

PULL CABLE: This cable conducts the pulling force of the solenoid to the puller bars via the upper and lower pulley assemblies. It is made of flexible metal with a nylon coating. NEVER PINCH OR DISTORT THE CABLE. The cable is terminated with crimped-on clamps or 'swages'.

The top is the metal plate on which is mounted the panels, cover plate, etc. Two other items are the cradles which support and hold the gas cylinder and the cover stops to which the plastic cover is secured. The base contains the transformers, circuit board and pull solenoid assembly.

SECTION 3

FRONT PANEL DESCRIPTION AND OPERATION

The aim of this section is to provide the user with the information necessary to operate the P-80/PC. It begins with some important definitions and descriptions of the front panel controls.

DEFINITIONS

Program: A program consists of one or more cycles, which when executed in sequence will 'pull' the capillary glass inserted in the instrument. A program can be up to 16 cycles in length.

Cycle: A cycle consists of a HEAT VALUE (range 000 TO 999), a PULL value (range 000 to 255), a VEL.(VELOCITY) value (range 000 to 255) and TIME value (range 000 to 255).

Loop: A loop consists of one or more cycles that are repeated by the instrument until glass separation is achieved.

Ramp Test: A program, resident in the ROM, designed to facilitate program alterations when it is necessary to change filaments. This test is discussed in subsequent text.

FRONT PANEL CONTROLS

Power: When pushed to the up position, this switch applies power to the instrument.

Reset: When momentarily pushed, this switch resets the microprocessor to an initialized condition. It is used to change from one program to another, and in other circumstances as described below.

Keyboard: There are three 'groupings' of keys on the keypad: numerical/decision, editing and control. They function as follows:

Numerical/Decision (0-9): These keys are used to enter the number of the program being chosen, the various values for HEAT, PULL etc. and to make yes/no (1/0) decisions in certain situations.

Editing (CLR, ENT, NEXT, LAST): These keys are used for entering, deleting and editing programs. They allow one to move forward and back through a program, enter new values, and clear out unwanted values. In addition, the CLR key is the access key to the Ramp Test. The function of the various keys will be explained in the context of instrument operation in subsequent text. Control (PULL, STOP): These keys control the initiation and cessation of program execution and control of the Ramp Test.

OPERATION

Apply power to the instrument. After an automatic 'power on' reset, the display will appear as follows:

WHAT PROGRAM DO YOU WANT TO USE (0-9)

he P-80/PC is shipped with two programs already stored in memory. Program 0 is for a micropipette in borosilicate or hard glass. Program 1 is a patch-type pipette in borosilicate or hard glass. Programs 0 and 1 were written for 1mm OD/.5mm ID borosilicate glass (and a gas flow micrometer value of 100). Assuming that 1mm OD/.5mm ID glass is available, proceed in the following manner. Raise the cover and load a piece of glass into position. This is best done by loosening the glass clamp on the right or left puller bar and sliding the capillary through the clamp until it projects about 1 centimeter beyond the clamp. Release both puller bars and pull them to the center until they stop. Now slide the glass through the filament and into the glass clamp on the puller bar and tighten the two wing nuts on the glass clamps. The wing nuts can be tightened quite a bit without breaking the glass, but a tremendous amount of force is unnecessary. In this particular instance the user may wish to leave the top in the up position in order to watch the pulling process; however, in normal use the cover should be down whenever a pipette is being pulled. Now press the number 1 on the keypad; cycles 1 and 2 of program 1 will appear. Press the PULL key and the puller will execute program 1. Whether one obtains a patch-type pipette or not depends on several factors. The programs contained in memory were written for a particular environment (ambient temperature and humidity) and type of glass. Remove the pulled pipettes from the glass clamps, close the cover and toggle RESET.

Once again the display shows the sign on message:

WHAT PROGRAM DO YOU WISH TO USE (0-9)

At this point there are three options: 1) choose a program, load glass and execute the pull; 2) create a new program or edit an old one; 3) run the Ramp Test. The user should now create a program. (please note that the programs that will be written in the following text are not meant to pull pipettes, but are intended as an exercise to help develop an understanding of the programming process. DO NOT USE HEAT SETTINGS GREATER THAN THOSE FOUND IN PROGRAMS 0 AND 1 UNTIL YOU UNDERSTAND THE SIGNIFICANCE OF THE HEAT VALUES.)

First press a key other than 0 or 1. The display should come up with no values for HEAT, PULL, etc. For example, if 3 were pressed, the display would look like this:

3 01 HEAT= PULL= VEL.= TIME=

3 02 HEAT= PULL= VEL.= TIME=

with the cursor blinking in the leftmost position of the heat value on the first line.

If there are already numbers or symbols entered as program values, make sure that this program was not entered by another user of the puller. Unused program areas are usually cleared before a puller is shipped, but occasionally random values or test program values are inadvertently left in memory. Since the program values for heat may be sufficient to damage the heater filament, we recommend that unused programs be cleared completely before proceeding. The clear function is outlined below under the heading 'CLEAR'.

Press a series of three numbers such as '333'. Notice that these numbers are loaded in the HEAT value, and that the cursor has moved on to the PULL value. IF THREE NUMBERS ARE PRESSED WHEN LOADING A VALUE, THAT VALUE IS AUTOMATICALLY ENTERED, AND THE CURSOR MOVES TO THE NEXT POSITION. If three digits are entered for TIME, the cursor moves to the next HEAT value and the display 'scrolls' to the next cycle. For PULL now enter two digits such as 10. In order to complete the entry, press the ENT key. The two digit entry is right justified and the cursor moves to the next position. IF TWO OR ONE NUMBER(S) ARE/IS PRESSED WHEN LOADING A VALUE, THE ENTRY MUST BE COMPLETED BY PRESSING THE ENT(ENTER) KEY; THE CURSOR MOVES TO THE NEXT POSITION. Continue by entering a value for VEL. (ie 30). Enter a value for TIME (IE. 80) and press ENT. Enter another set of values, such as 320 for HEAT, 60 for PULL, 10 for VELO, and 80 for TIME. Currently the cursor is placed in the HEAT value for cycle 03. Press LAST; this will move the display back to the 'last' cycle. Press LAST again, and the display will look as follows;

3 01 HEAT = 333 PULL = 10 VEL. = 30 TIME = 80

3 02 HEAT = 320 PULL = 60 VEL. = 10 TIME = 80

Now press NEXT; the display scrolls up one line, and the cursor is on the 'next' line of the program. TO MOVE AROUND IN A PROGRAM FOR THE PURPOSES OF EDITING USE THE ENT, NEXT AND LAST KEYS. Remember, the cursor only moves to the right. If the cursor were in the PULL value position on line 01 above, and one wished to change the HEAT value; press ENT three times to arrive at the HEAT value on line 02, and then press LAST. The cursor will be in the correct position for entering the new value. PRESSING ENT WITHOUT ENTERING A VALUE WILL CAUSE THE CURSOR TO MOVE TO THE NEXT POSITION WITHOUT CHANGING THE VALUE AT THE CURSOR'S PREVIOUS LOCATION.

It is appropriate at this time to define the units attached to HEAT, PULL etc.

HEAT: A change of one unit (ie 333 to 334) represents a change of 50 milliamps in the current through the filament. Generally changes will be made in steps of about 5 units since in most cases smaller changes will have no effect.

PULL: A change of one unit represents a change of 4 milliamps in the current through the pull solenoid. In the case of pull strength useful changes are 10 units or more to see an effect.

VELO: One unit represents a change of one or more millivolts of transducer output depending on the transducer being used. Useful values for velocity range from 10 to 100 with the lower values being used for patch and injection pipettes and higher values for micropipettes.

TIME: One unit represents .5 milliseconds if the velocity value is 1 or greater. One unit represents 10 milliseconds in the VEL.(VELOCITY) = 0 mode. See subsequent text for further details.

Assume for the moment that the two line program entered above, if executed, caused a glass capillary to stretch but did not cause the glass to separate. What happens next? The puller is 'aware' of the fact that the glass has not separated, and will go back to line 01 of the program and try again; in effect it begins 'looping'. It will continue to do so until the glass separates. This looping capability is very useful. For example, consider the following two-line program:

3 01 HEAT = 350 PULL = 10 VEL. = 25 TIME = 80

3 02 HEAT = 320 PULL =   VEL. = 10 TIME =

Assume that, after loading a piece of glass into the puller and executing the program above, that the filament came on five times before the glass separated. This indicates that the puller was into the third time through the program (looping) when the glass separated. Further more, assume that the result of the pull wasn't quite the pipette profile being sought; possibly because there was too much heat on the last pull. Then one might construct a new program that read like so:

3 01 HEAT = 350 PULL = 10 VEL. = 25 TIME = 80

3 02 HEAT = 320 PULL =   VEL. = 10 TIME =

3 03 HEAT = 350 PULL = 10 VEL. = 25 TIME = 80

3 04 HEAT = 320 PULL =   VEL. = 10 TIME =

3 05 HEAT = 300 PULL = 10 VEL. = 25 TIME = 80

Note the reduction in the heat value in cycle 05. This illustrates how the looping capability can be used to create a multi-step program designed to pull in one program execution. At the completion of the pull the puller will report on the number of times it looped and which line it was on at the time the pull took place. In the two line example above it would report the following.

PROGRAM LOOPED 3 TIMES

LAST CYCLE USED WAS LINE 1

The display then shows the first line of the program, and is ready for another pull. NOTE: IF ONE HAS FINISHED EDITING A PROGRAM AND WISHES TO EXECUTE IT, THE DISPLAY DOES NOT HAVE TO BE RETURNED TO LINE 01 BEFORE EXECUTION. PRESSING THE PULL KEY ASSUMES THAT EXECUTION IS TO BEGIN AT THE FIRST LINE OF THE PROGRAM.

Another user decides to use the program above, but the glass he/she is using is slightly different (thinner wall, different composition). Three possibilities exist: 1) glass pulls in same number of cycles; 2) glass pulls in less cycles, or; 3) glass pulls in more cycles. IF A PULL OCCURS AT A CYCLE OTHER THAN THE LAST ONE IN THE PROGRAM, THE CURSOR WILL BE LOCATED ON THAT LINE AT WHICH THE PULL TOOK PLACE. In the case of our program above, if the pull took place during the cycle on line 03, then the cursor would be on line 03. Likewise, if the pull takes place on a cycle as the puller begins to loop (because pull did not occur by line 05) the cursor will be sitting at that location.

A value of '0' entered for either TIME or VEL.(VELOCITY) has special meaning:

If VEL. = 0; PULL is disabled (= 0); HEAT is on at programmed value for duration of TIME programmed (10 milliseconds/unit) and cycle executes only once, no looping. This allows the one to use the puller as a 'polisher' for patch-type pipettes and the like.

If TIME = 0 and VEL. is _not_ equal to 0; the gas solenoid is disabled (no active or gas cooling). This allows the pulling of special pipette shapes. Most often used to pull long tube-like shapes such as are used for microinjection or micro-perfusion.

If both TIME and VEL.(VELOCITY) are _not_ equal to zero; then the value of TIME is the delay _from_ the simultaneous turning off of the filament and turning on of the gas solenoid, _to_ the turning on of the hard or solenoid pull. Increasing the value of TIME will increase the effectiveness of the active cooling prior to the hard pull. It is, to a degree, the equivalent of changing the micrometer flow valve.

There is always the possibility that the puller will be given a set of values which 'stall' its operation. An example might be where the HEAT value has not been set high enough to melt the glass, thus the glass can not be pulled and no velocity can be achieved. If it appears that a situation of this type has arisen, press the STOP key. This action aborts program execution and allows editing to take place. One could press RESET, but this requires that the program number be reentered.

Finally, it should be mentioned that all programs entered into memory (to a maximum of ten) remain there even after the power is turned off or the RESET switch is toggled. A special memory 'chip' that carries its own battery back-up will retain stored information for as long as ten years without power being applied to the instrument. Miracle that this is, it is strongly suggested that one keep a written record of programs in case of unexpected difficulties.

CLEAR: When a new program is being entered into memory in an area occupied by another program it is helpful to be able to 'clean out' the old program. Also, it may be desirable to

remove all the values from the last cycles of a long multi-cycle program to allow for fine tuning of these final cycles. This clearing of program values is accomplished by the CLR key.

The CLR key sets all values to 0 from the line on which the cursor is located to the end of the program. Thus, if the cursor is on line 05 of a seven line program and your response to the query:

DO YOU WISH TO CLEAR ALL VALUES FROM THE PRESENT

LINE TO THE END OF THE PROGRAM YES = 1, NO = 0

is a '1'. Only the values up through line 04 will remain intact. Clearing out a whole program simply requires that the cursor be on line 01 before the 'yes' response.

RAMP TEST

The 'no' or '0' response to the above question provides access to the most unique feature of the P-80/PC, the Ramp Test. If one answers 'no' to the question above, the following display appears:

DO YOU WANT TO PERFORM A TEST?

NO = 0, RAMP = 1

A '0' response returns the user to the current program. A '1' response enters the Ramp Test. The next display after entering '1' is:

LOAD GLASS, CLAMP AND PRESS PULL

A length of capillary glass is loaded, the cover lowered and the PULL key depressed. On the display a number will be seen to be incrementing at the rate of 10 units per second. Events take place as follows: 1) the puller increments the heat at the rate of ten units per second; 2) when the heat output begins to soften the glass, the puller bars begin to move apart. When a certain velocity (the value of which is stored in ROM) is achieved the heat is turned off and the ramp test value is shown on the display. In order to run the Ramp Test several times, it is necessary to press RESET, choose a program number (any number will suffice), answer 'no' to clearing values, and 'yes' to Ramp Test. Glass may then be loaded and the test run again.

Recall that one has been programming the P-80/PC based on the characteristics of the filament that is installed. Since no two filaments are exactly alike, there must be some way to adjust programs when a filament wears out or is damaged and must be replaced. The answer is the Ramp Test. One of the first actions that should be taken is to run the Ramp Test with the glass that will be used for pipette fabrication. An average of several tests gives a number that relates that particular filament to

that particular batch of glass. If the filament must be changed, or a new batch of glass is obtained; the Ramp Test can be used to establish a new Ramp Test value to act as a guideline for adjusting program values. It is necessary for the user to keep track of Ramp Test values.

SECTION 4

OPERATION

A. HEATER SETTINGS (FILAMENT HEAT)

CAUTION! Because of the large power reserve of the regulated heater power supply, it is very easy to burn out the filament if the heater value is set too high. The recommended starting heater value is the ramp test value.

At a heat setting of ramp value plus 15, a 1mm O.D., 0.50mm I.D. glass capillary tube should pull in 4 to 6 seconds after the start button is pressed. If the pull takes longer than eight seconds, and you are trying to pull a fine micropipette, increase the heat value by about five. Then try pulling electrodes until the pull takes place in less than eight seconds after the start button is pushed.

If the pull occurs in less than three seconds after you start, decrease the heat value by five. For 2mm O.D. tubing, the pull should occur between 15 and 25 seconds after the start. Make corrections as outlined for the smaller tubing.

The position of the glass within the filament will also affect the time it takes to pull an electrode. When using a trough filament the glass should be about .5mm above the bottom of the filament and centered front to back. The position of the glass with respect to the filament may easily be adjusted with the two eccentrics (A and A' in figure 2). The two locking screws B and B' should be loosened before adjusting the two eccentrics. In the case of a box filament the glass should be in the center of the filament.

The heat setting can also affect the length and size of the tip. Higher heat settings will give longer and finer tips. A heat value of the Ramp Test value plus 15 will generally give a very fine tip.

If should be noted that at high heat settings (filament white hot) the filament life is greatly reduced. It is suggested that a setting of ramp value plus 15 be used initially and electrode length be controlled by gas valve adjustment.

For patch-pipettes and injection pipettes a good starting point is the ramp test value. The time for the first pull will be in the 10 to 20 second range.

B. PULL STRENGTH ADJUSTMENT

Low values of pull strength settings in the range of 40-75 will give larger tips, while settings between 150-250 give the smallest tips. The pull strength can be set to any value desired with no danger of damaging the instrument. The hard pull is turned after the velocity reaches the trip value programmed for the cycle and after the TIME value has elapsed. If the velocity value was greater than 0 the TIME value determines a delay from

when the filament is turned off/nitrogen is turned on to when the hard pull begins. This time is in .5 millisecond units and a setting of 80 (40 milliseconds) is recommended as an optimum value for fine tips.

C. VELOCITY ADJUSTMENT (TRIP POINT)

The velocity value is generally between 60 and 120 for micropipettes and 10 to 30 for patch-pipettes. This value is related to the speed at which the two electrode carriers are moving. The lower the velocity value the slower the speed of the carriers at which the trip point will occur. At that time the heat will be turned off and the nitrogen will be turned on. After a delay determined by the time value the hard pull is turned on.

D. GAS FLOW

The gas flow control valve is located above and behind the filament. It is the primary means of adjusting the length of the electrode tip. It is a precision unit and will give reproducible results.

For a first try, set the control valve to a reading of 100. Increasing the gas flow will produce shorter tips, and, conversely, decreasing the flow will produce longer tips.

If the gas flow is decreased too much, the electrode will not form a tip. At air flow settings about five units below the value needed to form a tip, the glass will break and form tips of about one micron. At still lower settings, the glass will form a wispy fiber. The very finest tips for a given pull and heat will be formed at an air setting 10 units higher then where the 1 micron tip was formed. Long tips can be formed by using wider filaments or by using higher heat settings, and conversely still shorter tips can be formed by using narrower filaments or lower heats. Filaments narrower than 2mm can not form as fine a tip as the wider filaments.

Electrodes will not be formed if the gas flow is set too high.

E. CAPILLARY MOUNTING

We suggest the following method of inserting the glass capillary tubing into the carrier clamps, to prevent damage to the filament.

Use either the left or right carrier; move the carrier away from the filament until the carrier is latched by the spring clip. Open the capillary clamp; hold the glass tubing about two inches from one end, and with the two-inch end facing the filament, lower the glass into the clamp and tighten the clamp. The glass should now be in the groove with one end about 1/2-inch from the filament.

Release the spring clip latch and move the carrier toward the filament. If the filament is correctly positioned, the glass will pass through it. Hold the two carriers toward the center by

placing two fingers of one hand on the finger bars. Loosen the clamp holding the glass and slide the glass in its groove toward and into the other clamp groove. Center the glass and tighten both clamps.

F. HORIZONTAL TROUGH FILAMENT

The horizontal filament is quite easy to work with. This filament should be centered between the two clamps, and the air jet should be centered under the filament about 2mm below the filament.

When using the standard 3mm trough filament, the glass tubing should be positioned just above the filament and centered between the two sides. This position can be adjusted by using the two eccentric cams, located on the aluminum angle piece which holds the filament assembly.

Slightly loosening the two screws, which lock the filament assembly in place, the filament can be moved in relation to the glass tubing by turning the appropriate cam.

he heater filaments are easily replaced by loosening the two clamp screws holding the filament in place. Slide out the old filament, slip in a new one, and position it over the air jet. Then tighten the two screws.

G. ELECTRODE LENGTH

The length of the electrodes pulled can be varied, as previously stated, by decreasing the heat or by decreasing the air flow. It can be changed by using filaments of different widths. Widths of 1.5mm to 6mm trough filaments can be used. Electrodes pulled using a 1.5mm filament will be very short and will have large tips.

Tips of 1-2u can be formed using a 1.5mm filament, with low filament temperatures and weak pull strengths.

The tip size will decrease with increasing filament width until a width of 3mm is reached. Increasing the filament width beyond 3mm will produce longer tips with a more gradual taper (which may penetrate better in some cases). However, the tip will not be any smaller.

H. BOX FILAMENT

Another type of filament which can be used is the box type heater filament. The box configuration is particularly useful with thick wall or double-barreled glass, since the box filament delivers more heat to the glass. This results in faster heating without the necessity of increasing the temperature of the filament. (Note that the HEAT value must be increased in order to reach an operating temperature.) The box filament also heats the glass in a more symmetrical fashion than trough filaments, so that the pipettes produced tend to be more straight and more concentric than those pulled with a trough filament.

The box filament has two primary limitations. First, it requires more current to heat to a given temperature than the same size trough filament. Thus it is possible to use wider trough filaments without exceeding the maximum current capacity of the puller. Second, the box configuration reduces the cooling effect of the gas jet. For this reason the box filament is not recommended when very short pipettes are to be formed.

The air jet should be centered directly under the box filament. The glass capillary tubing should be centered within the box filament.

The optimal size of the box filament appears to be 3mm wide, 3mm high and 3mm deep. To produce short, large tips, a box filament of 1.5mm width, forming a box 2mm on each side, may produce more straight tips than a trough filament.

There is a size limitation on box filaments that can be used with the Model P-80PC electrode puller. Box filaments wider than 3 to 4mm may exceed the maximum filament heater current that the P-80PC can deliver, thus limiting the filament temperature.

MAINTENANCE

A. PULLEY ADJUSTMENT

The position of the two pulleys which carry the cables from the solenoid to the carriers is adjustable. This adjustment should be made only if the two electrodes formed from one pull are of quite different lengths. This inequality is generally caused by the jet not being aimed at the center of the filament but may also be caused by unequal cable tensions. (To avoid unnecessary cable adjustment, be certain that the air jet is correctly positioned before proceeding.) The adjustment is made by moving one or both of the pulleys to equalize the tension on the two cables. It should be explained at this point that there are two sets of stops in the system. There are the stops in the carrier slots against which the carriers rest, and a stop to prevent the solenoid from being pulled out of its housing. The adjustment of the pulleys must be made so that the carriers will still come up against their stops while the solenoid is not against its stop. The two cables should not be under tension when the carriers are against their stops, this is the position they would be in just before pulling an electrode.

You should be able to press on either cable between the carrier and the pulley and there should be about a 2mm deflection before the solenoid hits its stop. If the deflection is more or less, the pulley position should be changed. This is done by loosening the two screws above the pulley andturning the chrome eccentric cam to move the pulley in smallincrements until the two cables are of equal tension. If the carrier no longer stops against its stop in the slot, but stops against the cable, then the cam must be adjusted back until the carrier once more hits its stop. It is important that the carriers come up against their stops with no tension on the cables. If there is tension, the initial pull will depend on how tightly you hold the finger stops when the glass is clamped in the carriers. If this happens, the electrodes will not be consistent from pull to pull.

B. CABLE REPLACEMENT

If the cable breaks or gets a bad kink, it will have to be replaced. This is done as follows.

First, take off the plastic cover by removing the three screws which hold it in place. Now the two screws which hold the cover plate down must be removed. The cover plate is the aluminum plate on top of which the filament assembly sits, and below which the two cable wires can be seen coming out on their way to the outboard pulleys. With these two screws removed, the complete filament assembly, including the air solenoid and air micrometer, can be lifted up and moved back out of the way.

Next, with the power cord unplugged, remove the five screws which hold the front panel in place. Swing the top edge of the panel forward so that the panel is face down on the table. The solenoid assembly can now be seen.

The next step is to remove the brass slug on the top of the solenoid. It is held on by two hex screws. The brass slug should now slide up and off of the shaft of the solenoid. The old cable can now be slipped out of the slot in the brass slug. At this point note the path of the cable as the new cable will be strung the same way as the old. To remove the old cable, use a wire cutter to cut the cable near the electrode carriers. Now pull out the cable. Slip the swaged end of the new cable in the brass slug and replace the brass slug. Be sure to get the hex screws tight. Both wires go through the first guide, and then each wire must be fed through a pulley and its accompanying guides.

Using a small screwdriver, loosen the screw at the outboard end of the electrode carrier and remove the short piece of wire and its swage. Feed the wires through the outboard pulleys so that the wires lie across the electrode carriers.

For the next steps, the electrode carriers must be held in toward the center. This can be done with a rubber band around the two finger bars to bring both carriers in to the center. Loosen the two screws which lock down the two outboard pulleys and center the pulleys in their travel.

Now slip a swage on one of the wires. The wire must now be pulled on in order to lift the solenoid. With the solenoid against its upper stop, position the swage over the hole at the end of the electrode carrier and crimp the swage with a swaging tool. Using a wire cutter, cut the excess wire off and tighten the screw down on the wire. This most be done for each wire.

The cover plate and filament assembly can now be replaced. It may be necessary to readjust the filament position in relation to the glass tubing. The instructions for adjusting the outboard pulleys should now be followed to get the correct tension on the cables. This is somewhat easier to do with the front panel down, to see the relationship of the solenoid and its stop.

The final step is to replace the front panel. Be sure that no wires are pinched between the front panel and the cabinet.

SECTION 6

TROUBLE-SHOOTING

A. PIPETTE TIPS

PROBLEM: What glass should I use?

The type of glass and the wall ratio to O.D. (outside diameter) are two of the most important variables in controlling tip size. For example using program 1 which we used to form the pipette in the SEM micrograph included with this puller when shipped from Sutter Instruments; borosilicate glass with an O.D. of 1mm and an I.D. of .50 will give tips of .06 to .07 micron. Using the same settings borosilicate glass 1mm O.D. and .78mm I.D. will form tips of .1 to .12 micron. Aluminosilicate glass with an O.D. of 1mm and an I.D. of .58mm will form tips of .03 to .04 microns again with the same settings.

In general the thicker the wall in relation to the O.D. of the glass the finer the tip will be, and the thinner the wall the larger the tip will be. Thin wall glass will give the best results in most experiments as it will have the largest pore for a given tip size. This means it will have a lower resistance and will allow for easier injection of solutions. However in many cases with small cells the thin wall glass will not form tips fine enough to obtain good penetrations. In this case heavier wall glass must be used.

PROBLEM: The resistance of my pipettes is to low, how do I pull a higher resistance pipette?

The first point to note is that there is very little correlation between tip size and electrode resistance. Most of the resistance of a microelectrode is in the shank of the electrode behind the tip. Electrode tips which are .1 micron in diameter can vary in resistance from 20 Megohms to 1000 Megohms depending on the length of the electrode and what is used for the filling solution. If the same solution is used then resistance may give an indication of how well the electrode will penetrate a cell as the electrode with the higher resistance will probably have a longer shank and a smaller cone angle at the tip. This combination will aid in the penetration of cells where the cell is not a surface cell.

PROBLEM: OK but I still want a smaller tip than I am getting.

The first thing to try in most cases is to increase the heat value. This will generally decrease the tip size but it will also give a longer shank. If the higher resistance is not a problem this is generally the best solution. Continuing to increase the heat is not the final answer as to high a heat can lead to larger tips. In general with 1mm O.D. .5mm I.D. borosilicate glass the finest tips will be formed when the glass pulls in 4 to 5 seconds after starting the pull.

If the electrode is now too long and causes the resistance to go too high to pass the necessary current for example, then the next step is to increase the pull strength. In general a pull strength of 125 will give tips of less then .1 micron. Increasing the pull to 250 will reduce the tips size about 5-10%. We recommend a pull of about 150 in most cases.

The last major variable to adjust is the nitrogen flow. If in the case of 1 by .5 borosilicate glass the pull takes place in 4-8 seconds the tip size will not change with a change in the cooling flow. The only change will be in the length of the shank. If however the heat is such that the pull takes place in more then 8 seconds, increasing the flow will increase the tip size. An increase in gas flow will shorten the tip and a decrease will lengthen the shank.

PROBLEM: How do I increase the size of my patch-pipette?

The first thing to try is to reduce the heat. Try dropping the heat 5 units at a time to see if this will increase the size of the tips. If this does not work increase the air micrometer in units of 5. The pull should generally be set to 0 when pulling large tipped (1-10 micron) pipettes.

PROBLEM: The tips of my patch-pipettes vary in size from pull to pull.

This can happen when a pipette is formed in two or more loops. If the pipette is formed in three loops in one case and then on the next pull it forms in four loops the tips will not be the same. Adding one unit in the velocity value will in most cases cause the pipette to be formed in three loops or subtracting 1 unit should cause the pipette to form in 4 loops. It is always good technique when a program is developed that produces a desired pipette, to try increasing and decreasing the velocity value to be sure that you are in a stable region. The best procedure in developing a very reliable pipette program is to change the velocity value both up and down until the number of cycles to pull the pipette changes. Then pick a value halfway between for the final velocity value.

PROBLEM: I need to form an injection pipette with a 1 micron and 20 to 50 microns long. How do I do this?

Try a program in which the first two lines of the program have a pull value of 0 a velocity value of 10 to 30 a time setting of 80 and use the ramp value for the heat. The third line should have the same heat value, a pull value of 150, a velocity of 30 and the time should be 0.

The idea behind this program is to reduce the size of the glass on the first two cycles and then on the third cycle we give a hard pull with the air turned off. Normally if the air is turned off a long wisp will result, but since we have greatly reduced the size of the glass and with a very hard pull the glass will tend to separate when it is about 1 micron in diameter.

PROBLEM: The electrodes are bent. How do I make them pull straight?

This problem occurs most often when using the trough filament. Going to a box type of filament will produce much more straight pipettes. The bend in the pipette has no effect on the pipettes tip and should cause no problems unless you are penetrating quite deep in the tissue with the electrode and you are aiming at a certain site. Then the bend may lead the pipette to the wrong area. The box filament is not a complete improvement on the trough filament as the gas flow is much less effective with the box filament, and you give up much of the length control that the gas gives with the trough filament.

PROBLEM: The filament does not light up when I press pull.

There are a number of possible reasons why this might happen. First look and see if the filament has burned out. In some cases it may be necessary to loosen the screws holding the filament in place as a very fine break may be hard to see. If the filament is OK, try running the ramp test and see what happens. If you have just changed the filament it is quite possible that the new filament needs a very different heat value than what you have been using. It is always a good idea to run the ramp test each time you change the filament. If you run the ramp test and the heat value reaches 999 without the filament heating up check the screws holding the filament in place and if they are tight then check the two nuts connecting the filament wires to the posts in back of the filament block. If these are tight then the problem is probably on the circuit board.

PROBLEM: One electrode is much longer then the other electrode.

This is caused by one of two things. First check the tension on the two cables as explained in the maintenance section. If the cables have the same tension then the gas jet must be aimed more at one side of the filament than the other. This can be corrected by moving the gas jet until both sides are the same length.

B. ELECTRONICS SYSTEM

The P-80/PC micropipette puller is controlled by a Z-80 microprocessor. Three digital to analog converters control the heat, pull and velocity values. The heat power supply is a precision constant current switching unit which will vary less than 10 millamperes with a plus or minus 10% change in the ac line current. The pull supply is a constant current DC power supply. The velocity trip point is set by a D-A converter. The output of the velocity transducer is compared to the output of the velocity D-A to determine when the trip velocity is reached.

CAUTION: DANGEROUS POTENTIALS EXIST INSIDE THIS INSTRUMENT. SERVICE SHOULD BE PERFORMED ONLY BY QUALIFIED PERSONNEL. THIS INSTRUMENT SHOULD BE UNPLUGGED FROM ITS POWER SOURCE WHEN ANY ADJUSTMENTS OR REPAIRS ARE MADE.

TROUBLE-SHOOTING

PROBLEM: The shape and resistance of the pipette changes from pull to pull.

 a. In most cases this is due to one or both of the cables to the pipette carriers being setup to tight. If the cable is adjusted so that the carrier can't come against the stop in the slot in the center of the pipette carrier. In this case the initial pull tension will depend on how hard the carriers are squeezed together when the glass clamps are tightened. To adjust, see the cable adjustment section.

b. A second possible cause of this problem is dirt on the carrier bars or bearings. In this case clean the carriers and bearings with a lint free tissue or cloth.

c. If the problem persists run the ramp test several times. If possible use one long piece of glass and move the glass over after each ramp test (turn the air adjustment up and the glass will not separate). If the ramp values are +/- 4 units or less the problem may be with the glass. If the values are greater than +/- 4 units call Sutter Instruments.

PROBLEM: Display blank, fan not on.

a. Check power cord and wall AC outlet

b. If unit is properly plugged-in and still does not work, remove power cord and check 3 AMP fuse. If the fuse has blown, suspect problems with the large transistors mounted on the heat-sink on the back of the cabinet.

c. If the fuse is still good, suspect the wiring harness.

PROBLEM: Display blank, fan on.

a. Check the 1/2 AMP fuse. If the fuse has blown suspect the main circuit board and trans-former T-3, a DMT 6-15.

b. If the fuse is still good, suspect a loose connection between the ribbon cable and display unit or the main circuit board.

c. If the connections are good check the various power supplies located on the main circuit board.

PROBLEM: Display shows a row of blocks.

a. The microprocessor has failed to properly initialize the display.

b. Press reset and the display should show the proper power-up message. Do not turn power off and then on rapidly, as this may cause improper power-up. Always allow at least 5 seconds before turning power back on.

c. If this problem recurs frequently suspect the reset timing capacitor, C 2. You may wish to replace C 2 with a slightly higher value such as 68 microF.

d. If pressing reset fails to produce the proper power-up message check pin 12 of U1 for a clock signal and check the address and data lines of U5 to see if the microprocessor is function-ing.

PROBLEM: Displayed program values are not correct.

a. Make sure that values were not changed by another user.

b. Always write down the program values and the ramp test value and keep them in a secure place.

c. If values entered are not held when the power is turned off suspect the zero-power memory, U 6.

CIRCUIT BOARD REMOVAL

Unplug unit from power. Remove three screws that hold plastic cover in place; one on each side and one on the rear.

Remove two nuts and washers that retain heater wires to the filament holder and remove wires from posts. (Fig. 1, H).

Remove six screws that hold top to base; two along each side edge and two along back edge.

Raise up top, move it forward slightly and rest it atop cabinet supported by solenoid bracket and back edge (Fig. 1)

Reaching around the solenoid bracket, unplug the molex connectors at the front edge of the circuit board and the 26 pin cable. The top may now be lifted clear and set aside.

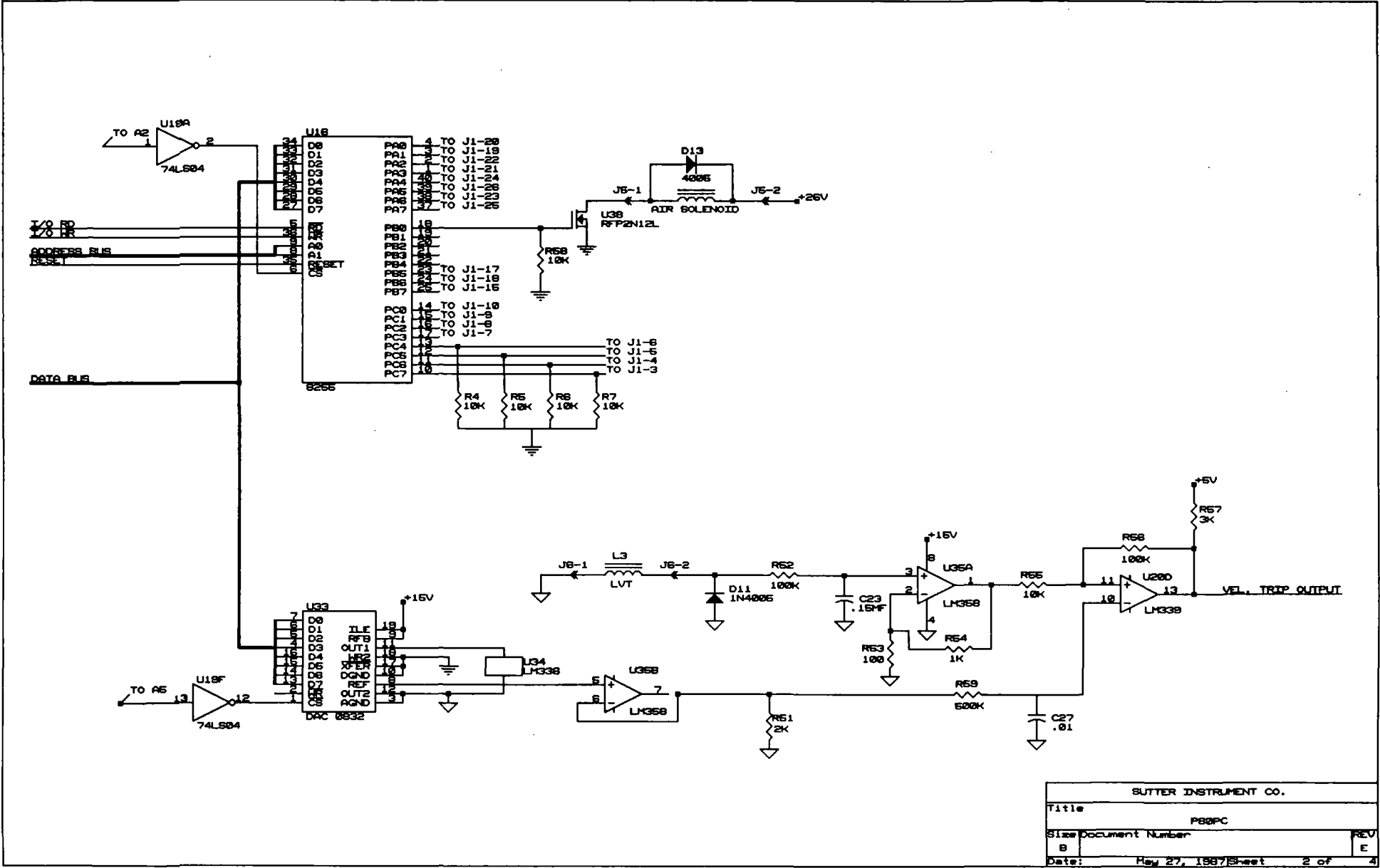Remove all other molex connectors.

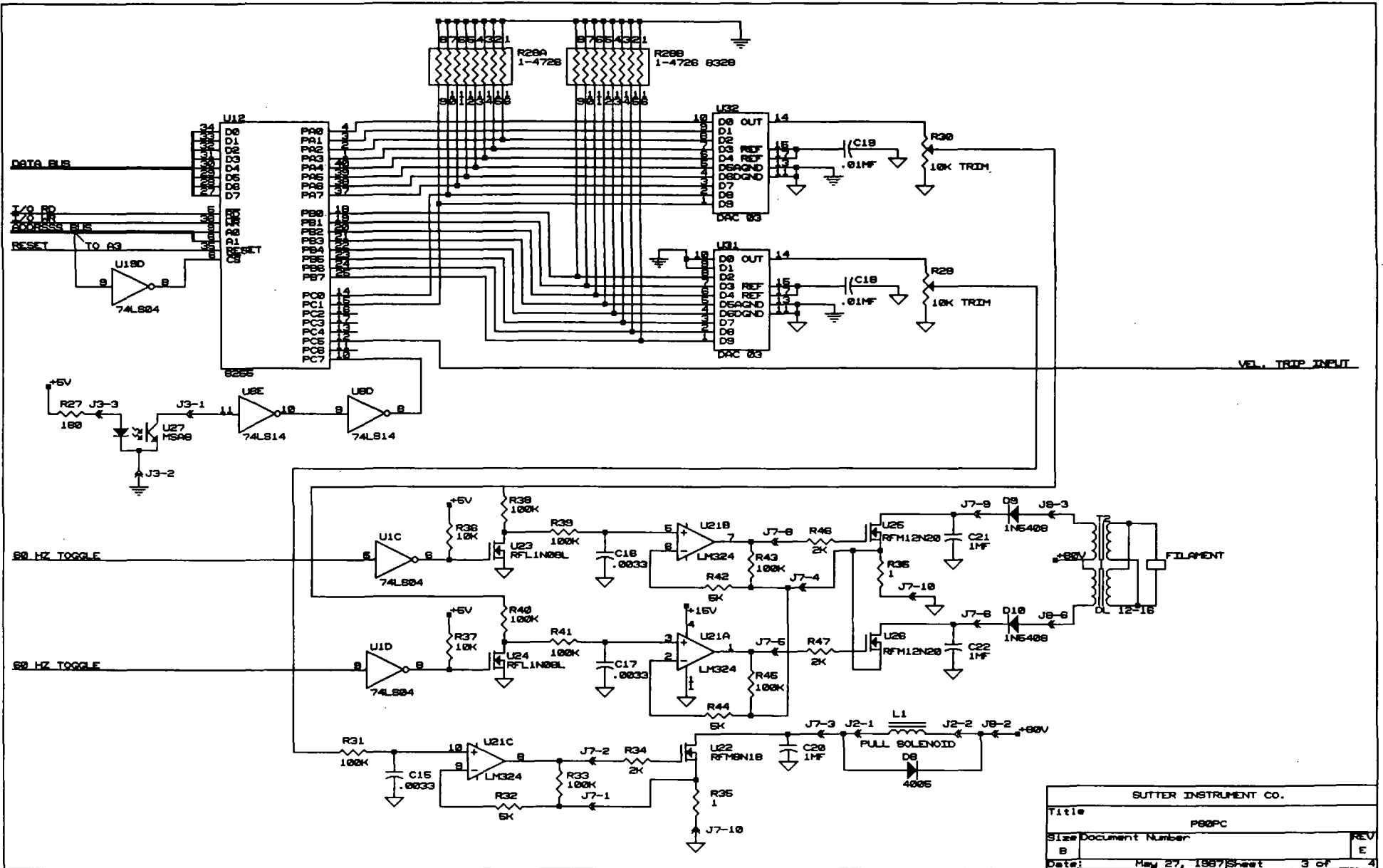Remove the eight plastic screws that hold down the circuit board (Fig. 2, locations E and E').

Lift the circuit board clear of the chassis and set aside.

Installation of the board is the reverse of the above procedure. Use extreme care in handling the connection from the velocity transducer. If the instructions are unclear, please contact us via phone or telex for clarification.

TO A2

U18A
1   2
74LS04

U16
34 D0
33 D1
32 D2
31 D3
30 D4
29 D5
28 D6
27 D7

PA0 4 TO J1-20
PA1 3 TO J1-19
PA2 2 TO J1-22
PA3 1 TO J1-21
PA4 40 TO J1-24
PA5 39 TO J1-26
PA6 38 TO J1-23
PA7 37 TO J1-25

I/O RD   5 RD
I/O WR   36 WR
ADDRESS BUS 9 A0
            8 A1
RESET   35 RESET
            6 CS

PB0 18
PB1 19
PB2 20
PB3 21
PB4 22 TO J1-17
PB5 23 TO J1-18
PB6 24 TO J1-16
PB7 25

PC0 14 TO J1-10
PC1 15 TO J1-9
PC2 16 TO J1-8
PC3 17 TO J1-7
PC4 13   TO J1-6
PC5 12   TO J1-5
PC6 11   TO J1-4
PC7 10   TO J1-3

8255

DATA BUS

D13
4006

J5-1   AIR SOLENOID   J5-2   +26V

U38
RFP2N12L

R58
10K

R4   R5   R6   R7
10K   10K   10K   10K

+5V

R67
3K

R58
100K

J6-1   L3   J6-2   R62   +15V   R65   11
         LVT   100K   8   U35A   1   13   10K   + U20D   13   VEL. TRIP OUTPUT
                                 3 -   LM358   10 - LM338
D11   C23   2 +
1N4006   .15MF   4
                                       R63   R64
                                       100   1K
U33
7 D0
6 D1   ILE   19
5 D2   RFB
4 D3   OUT1
16 D4   WR2
15 D5   XFER
14 D6   DGND   U34
13 D7   REF   LM338
            OUT2
TO A5   CS   AGND
13   12
U18F
74LS04   5 + U35B   7
                                  6 - LM358   R59
DAC 0832   500K   C27
                                                                   .01
                                  R51
                                  2K

+15V

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Title   P80PC
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SUTTER INSTRUMENT CO.

Title: P80PC

Size: B Document Number REV: E

Date: May 27, 1987 Sheet 3 of 4

P1
PLUG AC MALE

E
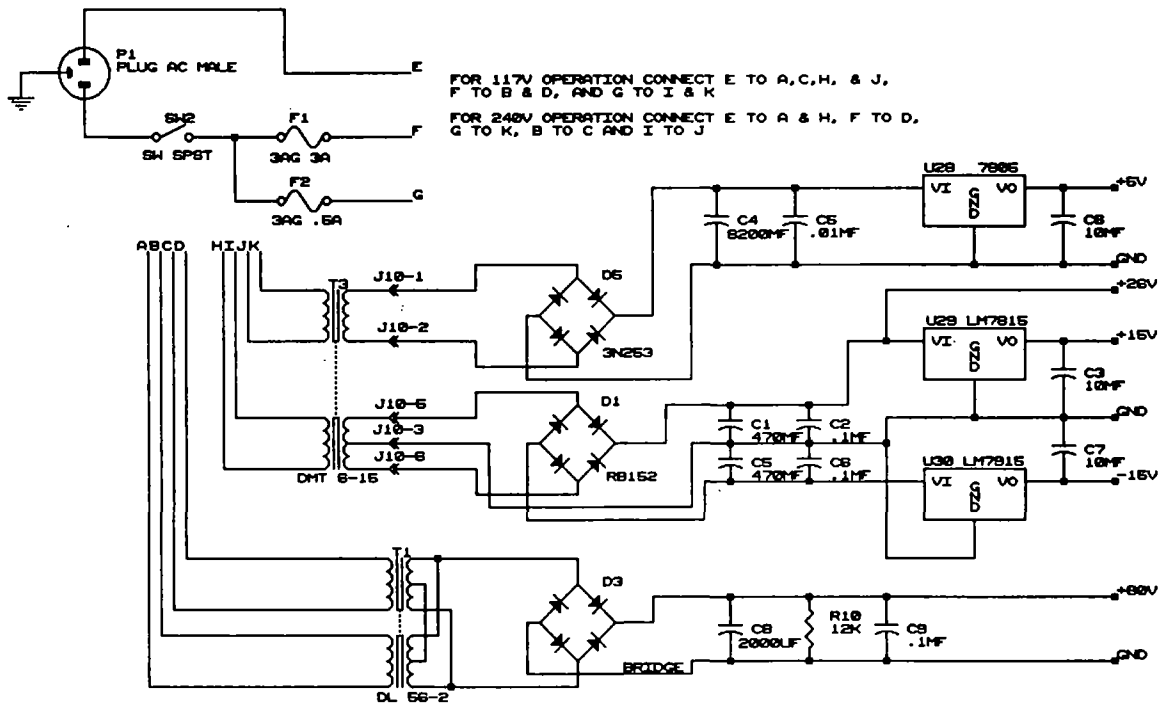
FOR 117V OPERATION CONNECT E TO A,C,H, & J,
F TO B & D, AND G TO I & K

FOR 240V OPERATION CONNECT E TO A & H, F TO D,
G TO K, B TO C AND I TO J

SW2
SW SPST

F1
3AG 3A

F

F2
3AG .5A

G

ABCD   HIJK

J10-1

J10-2

D5

3N253

U28 7805
VI GND VO

+5V

C4
8200MF

C5
.01MF

C6
10MF

GND

+20V

J10-5

J10-3

J10-6

D1

RB152

U29 LM7815
VI GND VO

+15V

C1
470MF

C2
.1MF

C3
10MF

GND

DMT 6-15

C5
470MF

C8
.1MF

U30 LM7815
VI GND VO

-15V

C7
10MF

D3

BRIDGE

C8
2000UF

R10
12K

C9
.1MF

+80V

GND

DL 56-2

COMMENTARY

NEW MICROELECTRODE TECHNIQUES FOR INTRACELLULAR WORK IN SMALL CELLS

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Abstract—Newly developed techniques are described for making both single and dual channel ultrafine electrodes, and for advancing these electrodes into small cells. Together with our previously described methods of electrode beveling, all of these techniques are found to be highly successful in rod outer segments of the toad retina, which average only about 6.0 μm in diameter. Hence the new techniques of making and advancing electrodes solve the main problems that have limited intracellular work in small cells. These techniques should also increase the efficiency of intracellular work in cells of all sizes. They thus offer promising possibilities for greatly expanding the preparations and problems that may be studied in neurophysiology.

CONTENTS

DEVELOPMENT of the micropipette electrode by LING & GERARD (1949) provided the technical basis for major advances in neurophysiology. Since then, however, continuing limitations of microelectrode techniques have largely determined which preparations and problems can be studied and some of the main trends in the field. In particular, high quality and systematic intracellular work has been confined almost entirely to large cells, because of difficulties in penetrating small cells and maintaining normal activity after penetration. These limitations have been especially severe in the vertebrate brain, spinal cord and retina, where the vast majority of cells is smaller than 20 μm in the critical dimension. Thus intracellular work in the vertebrate central nervous system has been devoted almost exclusively to selected large cells, and the search for especially large cells in lower vertebrates and invertebrates has become a well-estab-

lished trend. It seemed evident that the purposes of neurophysiology would be greatly advanced if the limitations of microelectrode techniques could be markedly reduced or abolished, so that cell size would no longer be a critical consideration. It was also evident that the technical problems could not be solved on a casual basis, so we undertook a systematic attack on these problems. This was done partly to expedite our own research in vertebrate photoreceptors, but mainly with the broader goal of removing technical barriers that have prevented intracellular neurophysiology from being applied systematically to small cells.

A MICROPIPETTE PULLER FOR FORMING ULTRAFINE TIPS WITH SHORT TAPERS

Requirements and background

We have previously reported methods for the rapid and precise beveling of Pyrex micropipettes with tip

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Abbreviation: SEM, scanning electron microscopy.

diameters as small as about 0.1 μm (BROWN & FLAMING, 1974; 1975). During that work the need became evident for an electrode puller that was more adequate than available pullers for the requirements of small cells. These requirements include the reliable making of very fine tips, while minimizing both the flexibility of the tip and its electrical resistance. Even the optimal application of electrode beveling to small cells requires the dependable formation of very fine tips, combined with the requisite stiffness for accurate and reliable beveling.

Most electrode pullers follow the two-stage plan of ALEXANDER & NASTUK (1953), whose design was followed closely in the puller-made by Industrial Science Associates. As illustrated by ALEXANDER & NASTUK (1953), the electrode tips are typically about 12 mm long: this length is from the beginning of the taper to the tip itself, and it cannot be reduced appreciably. With stringent attempts to pull small tips, we obtained unbeveled tips about 0.15 μm in diameter. as demonstrated by scanning electron microscopy (SEM). These tips were reasonably stiff and beveled well (BROWN & FLAMING, 1974), but were obtained only with considerable difficulty and low reliability. Also. in the outer segments of toad rods we find that electrode tips of about 0.15 μm diameter, even when minimally beveled. are too large for satisfactory results. This preparation requires ultrafine tips, which we define as tips having diameters of 0.10 μm or less. For such small tips the puller described by LIVINGSTON & DUGGAR (1934) is widely used. We have found this puller to provide tips as small as 0.05 μm, but the tapered portion is 25 mm or more in length. Hence the d.c. resistances are quite high, typically 300–400 MΩ when filled with 5 M K acetate. Also, the extreme flexibility of the tip makes these electrodes difficult to bevel and unsuitable for reaching target cells that are deeply buried in the brain or spinal cord.

It was first shown by LUX (1960) that cooling a glass capillary by an air stream while the tip is being formed can greatly shorten the tip. This principle was carried further by CHOWDHURY (1969), who directed two symmetrically opposed air jets to the forming electrode tip; this shortened the tip and yielded tip diameters down to about 0.05 μm. We initially built a puller following the description of Chowdhury (1969). and confirmed the validity of the principle, but results were disappointing because of the great variability between electrodes at specified settings. We have now developed a puller incorporating features found desirable to take advantage of the air jet principle, while also providing versatility and highly reliable results. In achieving these goals, many details proved important.

Description of micropipette puller

Figure 1 is an overall view of our puller, the major features of which will now be described. It is a two-stage horizontal puller, in which both electrode carriers are connected by stainless steel cables to a vertically oriented solenoid that is not visible in Fig. 1. The correct strength of slow pull is provided by the weight of the solenoid plunger and its attachments. It proved necessary to use a d.c. solenoid, to avoid variations of pull strength that occur when the strong pull can be activated at various phases of an alternating current. Smooth and consistent pulling motions are provided by each electrode carrier being mounted in V-groove ball bearing wheels. The outboard cable pulleys are adjustable laterally to give equal tension on both cables, and after this adjustment the two electrodes resulting from each pull are almost identical.

The heating element is a flat ribbon of 90% platinum and 10% iridium. This ribbon is 0.002 in. thick, about 2 mm wide, and formed into a loop about 3.5 mm in diameter. The heating element is powered through a Variac, preceded by a regulating transformer and heavy duty transformer that reduces the maximum voltage to 3 V. Provision is made for monitoring both voltage and current in the heater circuit. and we usually use values of about 1.8 V and 12 A for single barrel electrodes. Whenever the voltage required for a given current shows a significant upward trend, the heating element is replaced; this has proved helpful for maintaining reliable results over a long period.

In the air jet system a bottle of compressed nitrogen is used as the source of our 'air'. This minimizes oxidation of the heating filament and assures clean gas, with consistent low moisture content for control of its cooling effect. The gas pulse is formed by an electrical gas valve. The pressure from the nitrogen bottle has been kept at 50 lb/in², and gas flow during the pulse is controlled by a needle valve that is set by a micrometer. Figure 2 is a closeup view showing the heating filament and gas jets. Note that the nitrogen is delivered symmetrically through orifices positioned just above and below the heating element. These orifices are shaped like flame spreaders, the long axes of which coincide with the axis of the ribbon filament. This assures efficient delivery of gas to cool the heating filament rapidly, while the filament itself protects the forming electrode tip from being deformed by the gas currents. We found it both undesirable and unnecessary for the nitrogen to cool the electrode tip directly. Instead, the gas need only cool the filament rapidly to stop it from continuing to deliver a relatively large amount of heat while the tip is being formed. The heating element and gas jets are mounted on a single block that may be adjusted to center the heating element on various sizes of glass tubing. For prevention of tip bending, it also proved necessary to provide one-way catches that stop the electrode carriers from rebounding after striking their rubber dampers. The electrode tip is thus protected against re-entering the vicinity of the heating element while it is still delivering sufficient heat to deform the fine tip.

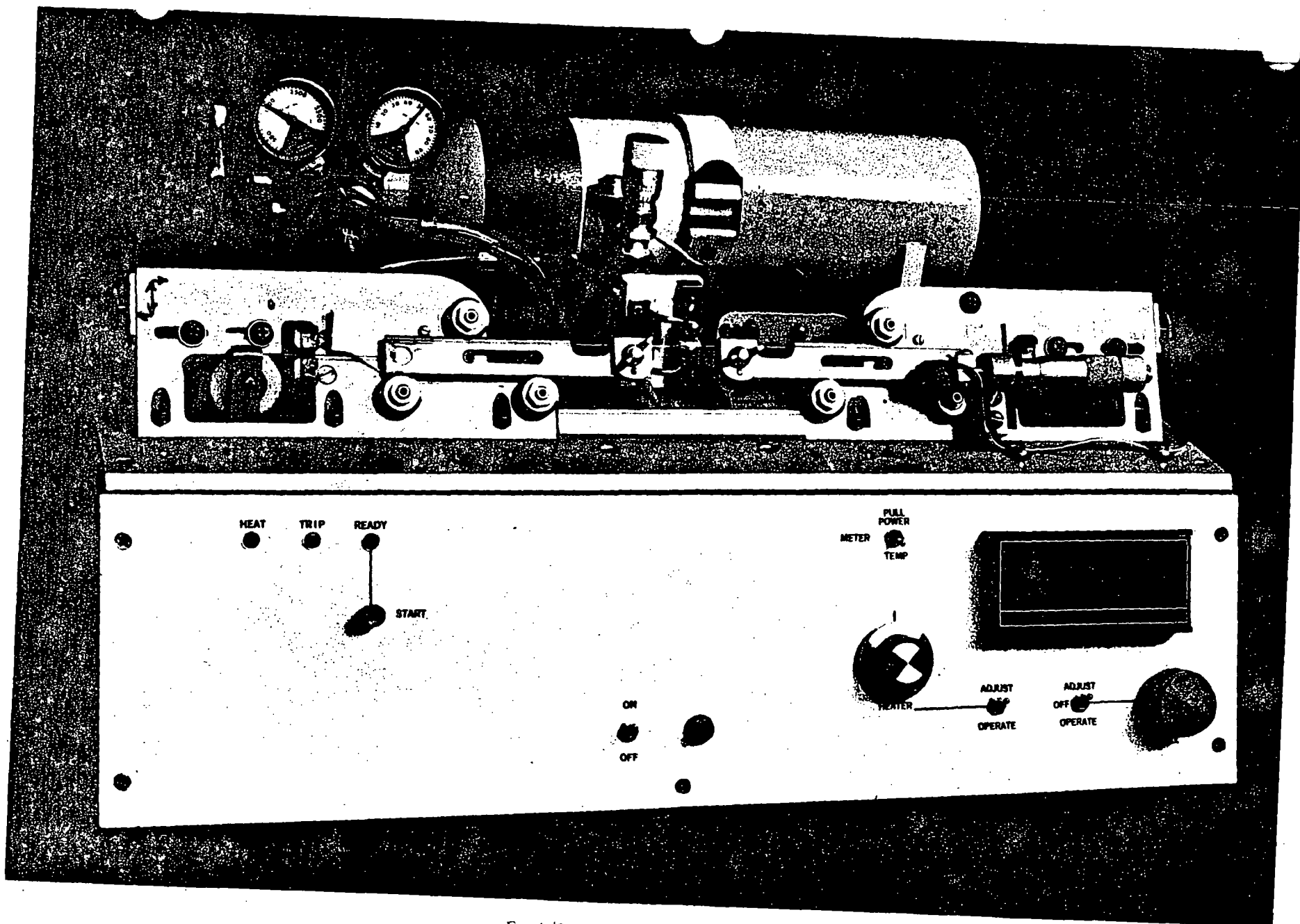For pulling electrodes a switch turns on the heat,
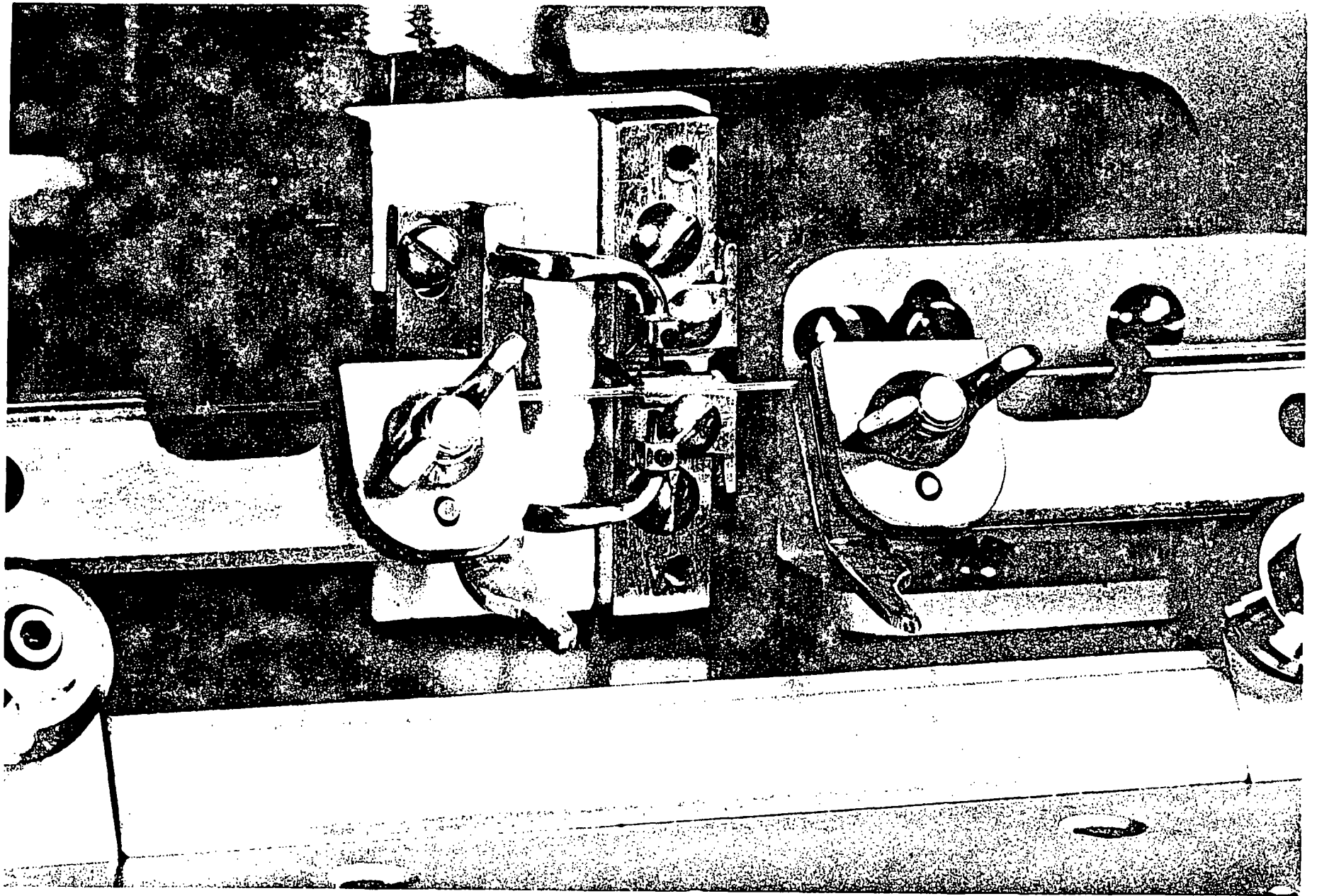
FIG. 1. Overall view of electrode puller.

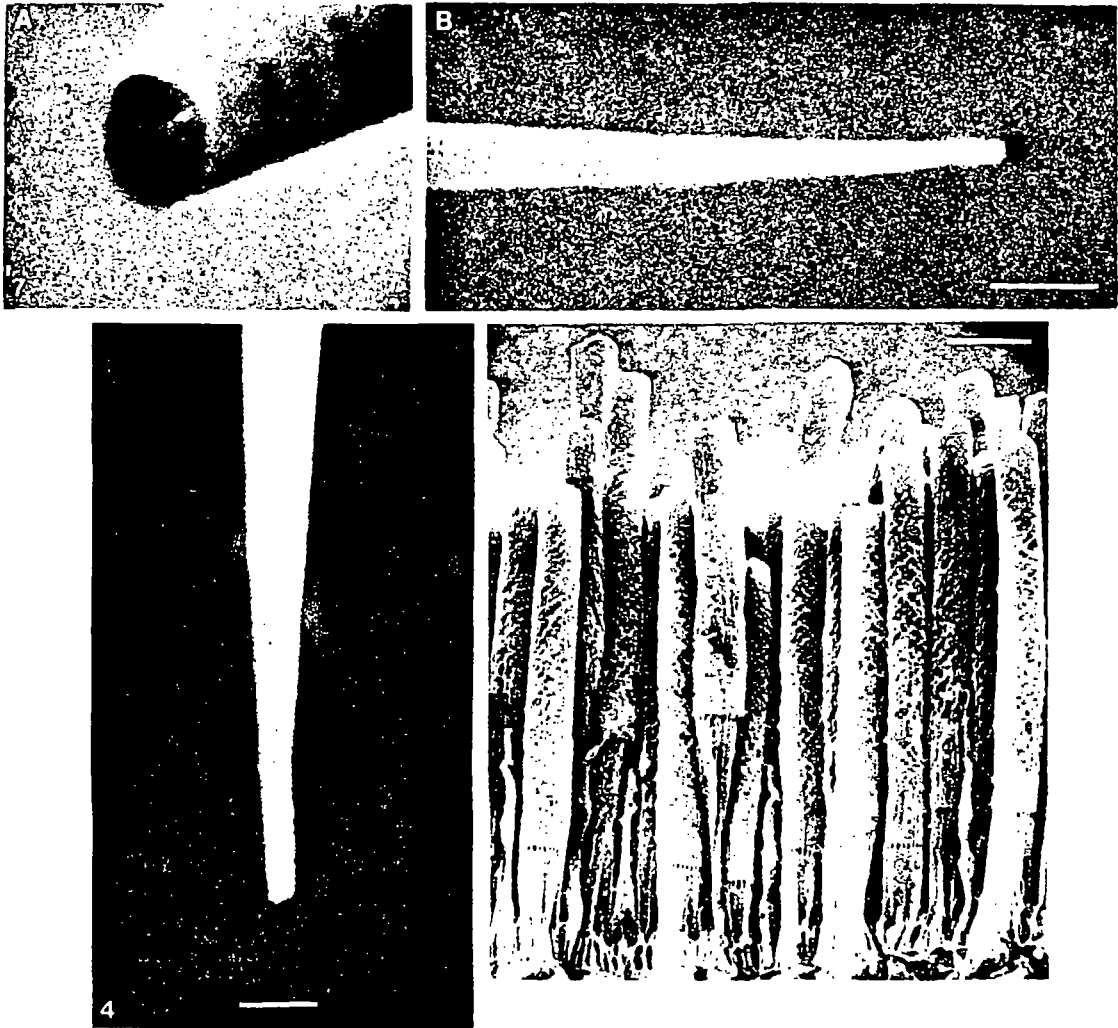FIG. 2. Closeup view of heating coil, jets and electrode clamps.

Fig. 4. Sample electrode tip photographed by scanning electron microscopy. Airflow setting of 75. Tip diameter measured 0.075 μm; after correcting for the gold coating, the true diameter was about 0.045 μm. The electrical resistance of the other member of this pair of electrodes was 115 MΩ.
Scale = 0.20 μm.

Fig. 7. Scanning electron micrographs of dual channel electrode tips formed from thick septum theta tubing. A: a larger tip broken to a diameter of about 0.4 μm, to illustrate that the relative cross-sectional dimensions of the theta tubing are maintained down to the tip. B: an ultrafine tip with a measured outside diameter of 0.085 μm; after subtracting the gold coating, the corrected diameter was 0.055 μm.
Scale = 0.40 μm.

Fig. 10. Receptor layer of the retina from the toad. Bufo marinus. The retina was fixed and cracked, and the exposed receptors were photographed by scanning electron microscopy, as described by STEIN-BERG (1973). The predominant receptors are red rods, with long columnar outer segments that are separated from the inner segments by a narrow gap crossed by many fine calycal processes. A few green rods may also be seen, which have shorter columnar outer segments, and inner segments that taper rapidly to a thin process. A few very slender cones may also be seen, interspersed among the bases of the rods. Scale = 10 μm.
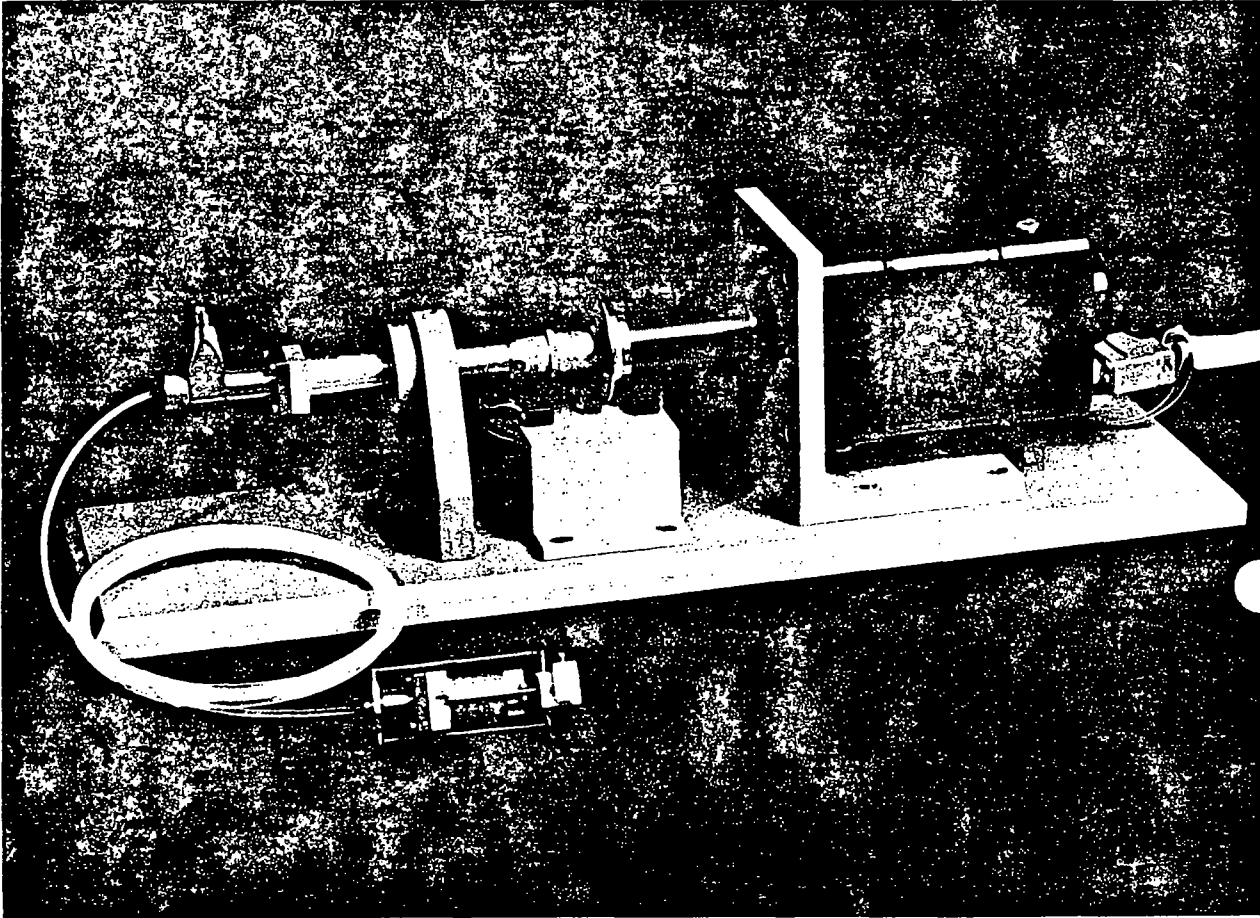
FIG. 8. Photograph of modifications to the Kopf hydraulic unit to make a high-speed stepping microdrive.

and the remainder of the pulling cycle is controlled automatically. When the capillary has been softened and drawn over a preset distance by the weak pull, a precision type of optical switch is activated. This optical switch is mounted on a micrometer with a non-rotating spindle, which is visible on the right side of Fig. 1. The length of the slow pull prior to activating the switch may thus be accurately controlled. The optical switch turns off the heat, initiates the gas pulse, and trips a delay circuit, at the end of which the pull solenoid is activated. It proved necessary to initiate the gas pulse about 40 ms before the strong pull, apparently because of the delay required for the gas pulse to cool the heating filament. During the strong pull the tip is formed in 5-8 ms, though the solenoid is activated for about 1 s. The duration of the gas pulse is arbitrarily set at about 200 ms. Hence the gas pulse normally lasts from about 40 ms before the strong pull to well after the electrode tip has been formed.

Characteristics of single barrel electrodes

Single barrel electrodes were formed from Pyrex 'Omega Dot' tubing of outside diameter 1.0 mm and inside diameter 0.5 mm, a solid glass fiber of 100 μm diameter having been fused to the entire length of the inner wall. To illustrate the effect of the gas pulse, electrodes were pulled with all values constant except the gas flow. The gas flow micrometer was set respectively at 55, 75, 100 and 150 thousandths, which gave

increasing gas flows. The low power micrographs of Fig. 3 show the length and overall configuration of the resulting electrodes. At the lowest gas flow the tip length from the beginning of the taper to the very tip was 13.0 mm. These electrodes were very similar in length to the electrodes formed by an Industrial Science Associates puller. With increasing gas flows the rapidity of taper increased and the tip length was reduced to about 6.0 mm. Within the illustrated range of gas flows, electrodes formed at any given gas flow were consistently similar in tip length and configuration. The largest gas flow of Fig. 3 cannot be exceeded appreciably, however, without obtaining considerable variability between electrodes.

Prior to measuring their d.c. resistance, electrodes were filled with 5 M K acetate by injection from the back, and allowed to.stand overnight, after which any bubbles in the shaft were removed by reinjection. Tips examined by SEM were not filled because the electrolyte was difficult to remove and often contaminated the tip. These electrode tips were mounted in either epoxy or solder glass (Corning No. 7570), then carefully cleaned. The detailed procedures in preparing electrode tips for high resolution SEM proved quite critical and are available upon request. To prevent electron charging during SEM observations, a conductive gold coating was deposited with a minimum thickness of 150 Å. All measurements of tip diameter must thus be corrected by subtracting at least 300 Å, or 0.03 μm.

Gas flow
setting

55

75

100

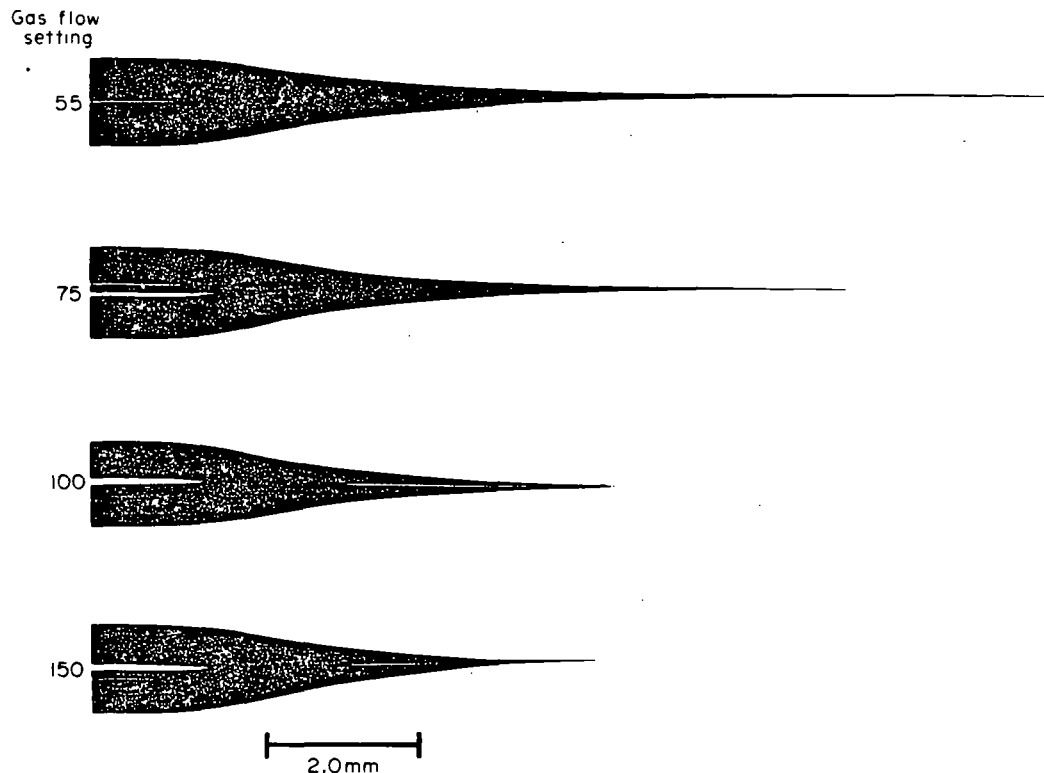150

|⊢———————— 2.0 mm ————————⊣|

Fig. 3. The length and configuration of electrodes, formed as described in the text, with four gas flow settings that delivered progressively greater amounts of gas during the nitrogen pulse.

Using the same puller settings as in Fig. 3, including the same four gas flows, we measured both tip diameter and electrode resistance. In some cases a single pair of electrodes yielded a measurement of tip size on one member of the pair, while the other could be used to measure electrode resistance. In such cases the electrode resistance associated with a given tip size and configuration should be determined with particular accuracy. Figure 4 shows an SEM photograph of a sample electrode pulled at a gas flow setting of 75. This electrode tip had a measured diameter of 0.075 μm, so the true diameter was 0.045 μm, and the electrical resistance of the other electrode of this pair was 115 MΩ. ·

Table 1 summarizes our results on tip length, tip diameter, and electrode resistance. Tip length was taken from Fig. 3. Note that measurements of tip size have been corrected for the gold coating. For both tip size and electrode resistance, the values given are averages, with the number of measurements entering each average given just afterwards in brackets. Surprisingly, Table 1 shows no clear change of tip diameter over the range of gas flows used. The average corrected tip diameter for all twenty-five electrodes in this series was 0.052 μm, and none of the tip diameters exceeded 0.07 μm. The smallest occasional tips had diameters of only 0.02 μm or 200 Å. It seems a meaningful comparison that this is only about three times the thickness of a cell membrane.

By contrast with tip diameter, electrode resistance decreased steadily and markedly with increasing gas flow (Table 1). Between the smallest and largest gas flow, the average electrode resistance decreased from 135 to 44 MΩ, a factor of about 3. Since this decreased electrode resistance was not related to tip size, it must have resulted from the more rapid taper of the shorter electrodes. At the highest gas flow setting in Table 1, the corrected average tip diameter was about 0.05 μm, while electrode resistance averaged 44 MΩ. This electrode resistance is very low for such fine tips.

If an electrode tip tapers too rapidly, in the region just behind the tip, the value of an ultrafine tip may be compromised by the electrode being too large where it finally lodges in the cell membrane. We have not encountered significant problems of this type, partly because the taper immediately behind the tip

was typically quite gradual, the cone angle of the tip in Fig. 4 measuring about 6°. In spite of this gradual initial taper, these ultrafine tips have demonstrated the requisite overall stiffness for beveling readily by our previously described techniques, as indicated by the gradual lowering of electrical resistance during beveling.

While the described puller forms ultrafine tips reliably, it has thus far not proved capable of forming reliable tips with diameters much in excess of 0.15 μm. This does not seem a significant disadvantage, partly because the ultrafine short tips should be preferable for many applications where larger tips have had to suffice. When larger tips are required, whether for greater strength, lower electrical resistance, or to facilitate injections, it seems desirable that these larger tips be obtained by beveling the ultrafine short tips. The advantages of the short taper may thus be retained, and the larger tip may be formed in a manner assuring its optimal sharpness.

As tip size increases, the difficulty of penetrating a cell with an unbeveled electrode probably increases approximately as the area of the tip, thus rising exponentially as the square of the tip diameter. Beveling forms an almost straight cutting edge, with which the difficulty of cell penetration should increase approximately linearly with tip diameter. Thus the advantage of beveling for penetrating cells should increase with tip size, and this seems confirmed by our observations. With ultrafine tips the advantage of beveling, from the standpoint of cell penetration, has proved significant but not critically important. Any desired tip diameter in the range of 0.1–0.5 μm may be obtained reliably by beveling to the requisite electrode resistance with 0.05 μm alumina, and electrodes with tip diameters mainly in the range of 0.2–0.3 μm showed dramatically improved intracellular performance after beveling (Brown & Flaming, 1974). Tips larger than 0.5 μm may be beveled reliably with diamond dust.

Summarized advantages of the new micropipette puller

This puller combines the advantages of ultrafine tips with the capability of forming quite short tips. Conveniently, gas flow may be used to vary tip length with little or no change of tip diameter. From the standpoint of absolute values, significantly smaller

TABLE 1. TIP LENGTH, TIP DIAMETER, AND ELECTRODE RESISTANCE AS A FUNCTION OF GAS FLOW DURING THE NITROGEN PULSE

Gasflow setting	Tip length (mm)	Electrode tip diameter (μm)*	Electrode resistance (MΩ)
55	13.0	0.052(8)	135(8)
75	10.0	0.049(8)	90(7)
100	8.0	0.063(4)	63(8)
150	6.0	0.048(5)	44(6)

* Tip diameters have been corrected for the thickness of the gold coating, as described in the text.

tips can be obtained than demonstrated with previous pullers. These tips can also be only half as long as the shortest tips provided by conventional two-stage pullers. With given settings and simple precautions, electrodes of given characteristics are formed with high reliability. In addition to single barrel electrodes, the heavier glass for our dual channel electrodes may also be used with little change in the required settings.

The short tips provided by this puller confer a variety of advantages. Compared with the Livingston type of puller, the electrode resistance of ultrafine tips may thus be reduced by at least a factor of 7. In addition to decreasing the noise level, this should improve the ease with which dyes and ions may be injected into cells, and our experience bears this out. The resulting stiffness of the shorter tips is very helpful in beveling, which improves the performance of even ultrafine tips, especially when injecting substances such as Procion yellow. The stiffness of these short tips should also be advantageous for penetrating cells, and for reaching target cells that require penetrating deeply or through tough tissue. The shorter tips likewise reduce capacitative coupling between the two sides of a double barrelled electrode, which has proved advantageous for our ultrafine dual channel micropipettes.

Finally, the combination of this puller with precision beveling techniques can reliably provide electrodes of any desired size ranging from the ultrafine tips formed by the puller to indefinitely large tips. All such electrodes should exhibit the advantages conferred by the rapidly tapering tip, combined with the extremely sharp tip formed by beveling. It appears that a versatile technique is thus provided for making high performance micropipette electrodes.

AN ULTRAFINE DUAL CHANNEL MICROPIPETTE ELECTRODE

Requirements

For many critical types of intracellular work, such as voltage clamping, two micropipette channels are required into the same cell. For cells that cannot be visualized, dual channel micropipettes are generally required. If the cells are also small, as are most cells of the retina and brain, these dual channel micropipettes present some difficult and conflicting requirements. Ideally these electrodes should be comparable to single channel micropipettes in penetrating small cells readily and providing stable normal responses. From this standpoint the dual channel tip should be provided within the ultrafine range of external tip diameter. With such small tips the electrical independence of the two channels tends to be strongly compromised, and this must be prevented. Also, with such fine channels the electrical resistance of each channel tends to be quite high, but must be held to a satisfactory upper limit. A dual channel micropipette electrode will now be described that is readily made, and

that meets all the above requirements, while also offering some additional advantages.

Dual channel electrodes formed from thick septum theta tubing

We first experimented with conventional dual channel electrodes formed by fusing two capillary tubes. When compared with single channel electrodes that were similarly pulled and beveled, and tested in the same types of photoreceptors, the single channel electrodes were highly satisfactory but the dual channel electrodes usually yielded smaller responses that deteriorated more rapidly. It seemed apparent that this resulted partly from the non-circular tip asymmetrically stretching the cell membrane, with the long axis of this stretching being undesirably great for small cells. Also, at the fusion of the two capillary tips, there are potential leakage channels between the inside and outside of the cell.

To obtain round tips we used 'theta' tubing, which contains a flat septum fused into a circular tube. Techniques for making such tubing have been described by KUMP & DEHN (1975). The theta tubing used initially was the standard type then supplied by W. R. Dehn (F & D Optical Systems, Inc., P.O. Box R 198, Spencerville, MD 20868). The septum of this tubing proved only about two-thirds as thick as the outer wall. Tips were pulled with this tubing and tested by injecting only one channel. Microscopic observation a few minutes later showed that not only was the injected channel filled to the tip, but the other channel was filling backward from the tip. Since fluid crossed between the two channels at the tip, of course the channels were not independent in either ionic composition or electrical characteristics. This undoubtedly resulted from the thin septum attenuating and disappearing slightly before the tip was formed by the thicker wall of the outer tubing.

It seemed likely that if the relative dimensions were reversed by making the septum thicker than the outer wall, considerable advantages would result. Theoretically the septum should then extend somewhat beyond the outer wall, forming a 'spear' in the center of the electrode that should improve cell penetration. It also seemed likely that this extension of the septum would effectively separate the ion pools utilized by the two electrode tips, thus decreasing the electrical coupling between the two channels.

Thick septum theta tubing proved more difficult to obtain, because of problems in fusing a thick septum with a flat edge into a round tube, but this was satisfactorily achieved by W. R. Dehn. Figure 5 shows a high contrast photograph of the cross section we now use and find satisfactory. Note that the septum is about twice as thick as the outer wall. The outside diameter of our tubing averages about 1.6 mm. Since this tubing is made by hand, the outside diameter varies from about 1.3–1.9 mm, but by far the greater portion of a given batch falls within 1.5–1.7 mm. All but a few of the smallest pieces had large enough
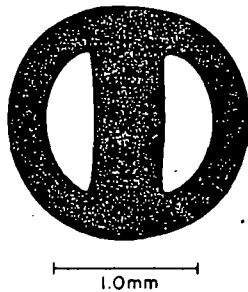
FIG. 5. High contrast photograph of cross-section of thick septum theta tubing used for dual channel electrodes.

channels to be filled by injection with 31 gauge stainless steel tubing, and even the largest theta tubing could be drawn by our puller.

Of course theta tubing requires a procedure to electrically separate the back ends of the two channels so that an independent contact can be made with the fluid in each channel. As shown schematically in Fig. 6, a high-speed Dremel tool was clamped and used to rotate a wheel that had diamond abrasive embedded in both edge and outer surface (Horico Diaflex No. 86 × SO, obtainable from suppliers of dental instruments). The theta tubing was held and advanced along the wheel so that the glass on one side of the septum was cut down about 12 mm. The septum was then surfaced with the diamond wheel, to obliterate any capillary channel that could remain if a portion of the outer wall were allowed to project out from the septum's surface. Fluid can also creep along the abraded surface of the septum, so the pro-
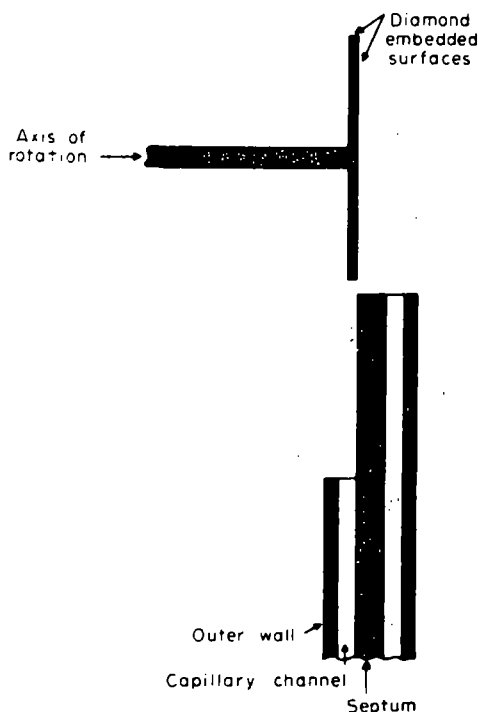

FIG. 6. Schematic illustration of method for separating the two channels at the back end of a dual channel electrode formed from theta tubing.

jecting portion of the longer channel was coated with a nonconductor material (Dow Corning No. 734 RTV). This silicone rubber compound provides a permanently nonconductive coating; being hydrophobic, it even resists being temporarily compromised by surface condensation. A coating of this material was placed all the way around the projecting glass of the longer channel; if applied only part of the way around, a capillary channel can occur where the material terminates, which destroys the value of the coating.

The requisite puller settings to provide tips of given size and length were little changed when shifting from 1.0 mm single channel tubing to the theta tubing. The heat setting increased, however, to soften the greater mass of glass in the theta tubing. Also, the gas flow had to be increased to cool this hotter filament fast enough so that the length of the tip was not thereby increased.

Characteristics of dual channel electrodes

Ultrafine tips were readily obtained with the thick septum theta glass. After correcting for the gold coating, the tip diameters usually measured about 0.07 μm. One of the smallest tips, photographed by SEM, is shown in Fig. 7A. Though not as fine as the smallest tips obtained from single channel tubing, the corrected diameter of the illustrated dual channel tip was only 0.055 μm.

In spite of strong efforts, it has not yet been possible to visualize the theoretical spear protruding from the tips of these electrodes. With ultrafine tips the very small apertures are filled by the gold coating, so end-on views give little information. In side views like Fig. 7A, the tip sometimes narrows distinctly near the end, as if a spear were being seen in edge view; if so, the gold coating seems to smooth out and obscure the details. Figure 7B shows a tip broken to a diameter of about 0.4 μm, which is sufficient to reveal the dual apertures. Note that the relative dimensions of the tubing are retained down to the tip, the septum remaining twice as thick as the outer wall.

For most test purposes each channel was injected with 5 M K acetate, which rapidly and reliably filled the tip. This rapid filling seems intrinsic to theta tubing, probably resulting from the capillary action at the sharp angles where the septum joins the outer wall. When only one channel of these electrodes was filled, no backward filling of the other tip could be seen under the microscope. The coupling resistance was measured conventionally as $R_c = E/I$, where R_c is the coupling resistance when a voltage, E, is measured through one channel as the result of passing a current, I, through the other channel. These measurements were made in the physiological saline bathing the retina, since coupling resistance rises after penetrating a cell. Control electrodes formed from thin septum theta tubing showed high coupling resistances, ranging from 5 to 35 MΩ. With thick septum

theta tubing, coupling resistances in the range of 100–200 KΩ were readily obtained. Our coupling resistances were thus as low as reported by WERBLIN (1975), who used similar measuring conditions, but who fused two capillary tubes together and then used beveling to separate the two tips by a distance of 0.5–1.5 μm. Since thick septum theta tubing provides similarly low coupling resistances with such fine tips, and without beveling, this result strongly supports the presence of the theoretical spear separating the tips; it likewise indicates the efficacy of this spear for lowering the coupling resistance.

The use of our puller to provide ultrafine tips combined with short tapers has special advantages with the dual channel theta glass. Since the ultrafine tip contains two channels, and also a thick septum, each channel must have a considerably smaller cross section than that of a single channel electrode. Hence it is particularly important to minimize tip length for reducing the resistance of each channel. The shortest tips tested measured about 6 mm from the beginning of the taper to the very tip. The resistance of each channel then averaged about 150 MΩ, which is satisfactorily low for most purposes. This compares with the average value of 44 MΩ that proved possible with single channel electrodes of similar tip length and slightly smaller outside tip diameter.

When compared with two capillaries fused together, the capacitative coupling between the channels of theta tubing must be greater per unit length of tubing because of the flat septum separating the two channels. Capacitative coupling was reduced, however, by the shortness of the tips. Compared with a thin septum, the thick septum is also helpful in this respect. As a consequence of these two factors, we have not encountered any significant problems resulting from capacitative coupling.

A HIGH-SPEED STEPPING HYDRAULIC MICRODRIVE

Features desired

While intracellular recording in small cells may be improved markedly by techniques of electrode making, techniques of electrode advancement are also crucial. Certain features of an electrode advancer are especially desirable. The electrode should move through the tissue by rapid steps, to improve cell penetration, and these steps should be short, to minimize the distance the cell is penetrated. Lateral electrode vibrations, which are usually associated with rapid electrode advances, must also be minimized because of their damaging effects upon the cell membrane. With rapid single step advancers, such as those using electromagnetic or piezoelectric principles, the step must be taken back and the electrode advanced by other means before the rapid step can be repeated (FISH, BRYAN, MCREYNOLDS & RIES, 1971; LASSEN & STEN-KNUDSEN, 1968; TUPPER & RIKMENSPOEL, 1969). Such advancers are most useful if the target cells may

be visualized, or if their locations are well known. It is thus desirable that the electrode be advanced by a train of steps, each of which has a good chance of penetrating any cell that the electrode has closely approached; this feature is especially helpful in masses of tissue, such as the central nervous system. Finally, it is desirable that the stepping rate can be relatively fast, with automatic stopping of the electrode upon cell penetration, in order to find cells quickly while also minimizing the damage resulting from cell penetration. An electrode advancer will now be described that offers all of these desired features.

High-speed modifications of the Kopf hydraulic microdrive

We began with the Kopf hydraulic microdrive (made by David Kopf Instruments, 7324 Elmo St. Tujunga, CA 91042). The hydraulic unit of this device, which may be used separately from the drive unit, is useful for preventing vibrations at the drive end from being transferred to the electrode. When this hydraulic unit is used with the Kopf stepping drive, a train of 1.0 μm steps is also provided. When tested for intracellular recording in the outer segments of toad rods, this stepping hydraulic drive yielded few cell penetrations. This was not surprising because the Kopf advancer was not designed for rapid steps, and the main limitations appeared to be in the drive unit. The stepping motor can operate at a maximum of only 200 steps/s, so the motor itself requires about 5 ms for a 1.0 μm advance. Also, if coupled directly to a standard fine-thread micrometer, this motor would give a single step of 2.5 μm. Hence the Kopf drive interposes gears to provide 1.0 μm advances. For rapid steps the inertia of these gears is undesirable.

The design of stepping motors has advanced greatly in recent years. The new motor chosen was a Model HDM-150-500-4-HS, made by Responsyn Products (USM Corp., Bldg. 3G, Sixth Road, Woburn Industrial Park, Woburn, MA 08101). When coupled directly to a standard fine-thread micrometer, this motor provides single steps of 1.0 μm, so the interposed gears may be eliminated. This motor also has a specially designed low inertia rotor and is relatively strong. These features permit stepping rates up to 1500/s, so each step is made in only about 2·3 ms. Though this motor required a new power supply, it was otherwise controlled by the Kopf electronic control unit. The method of coupling this motor to the driving micrometer is critical, because the axial motion of the micrometer must be taken up by the coupling. Our best solution to date is a metal bellows, designed to transmit torque without any significant rotary twisting of the bellows, while the bellows can change in length to accommodate the axial motion of the micrometer spindle (custom design No. SK-7160, obtained from Servometer Corporation, 501 Little Falls Road, Cedar Grove, NJ 07009). When attached to the motor and micrometer with light

magnesium adapters, this coupler minimized rotary inertia and introduced no detectable backlash. Figure 8 shows our drive system coupled to the Kopf hydraulic unit. Just forward of the coupling bellows is a disc that activates a microswitch at either extreme of the 25 mm of travel. These switches stop the motor and thus protect the instrument against overtravel.

In the hydraulic unit itself, the standard tube proved too small to reliably transfer a rapid fluid pulse. Tube length is also critical, since a longer tube decreases axial vibrations but reduces the velocity of an advancing step. These problems were solved by exchanging the standard tube for an 8 ft Teflon tube with respective inside and outside diameters of 0.060 and 0.120 in.

When a hydraulic unit was new, or when new rolling diaphragms had just been installed, it proved necessary to exercise the unit to obtain high-speed steps. This was apparently required to seat the diaphragms and to attain their maximum flexibility, and it was done by using a computer program to drive the unit back and forth for about 250 cycles. After this initial exercising, high-speed steps were obtained reliably. If the instrument was unused for a period of time, however, it proved necessary to renew the exercising.

Characteristics of high-speed steps

Little information has been provided on the movement characteristics of most electrode advancers, partly because convenient and inexpensive devices for measuring rapid movements have not been available. Such information is necessary to evaluate the critical details of the movement, and the requisite instrumentation is now available. We measured axial movement at the electrode end of the advancer by using a Unimeasure/80, a device utilizing the Hall effect (obtained from Unimeasure, 180 S. Lake Avenue, Pasadena, CA 91101). In this application movements of 0.1 μm could be resolved readily, and the response time for recording movements was not greater than 0.25 ms.

In Fig. 9 the control record was obtained with an unmodified Kopf stepping hydraulic microdrive. The maximum velocity attained during this 1.0 μm control step was about 0.27 μm/ms, and about 7 ms were required from onset to completion of the step. The same hydraulic unit was tested with the described high-speed modifications. Figure 9 shows that a single 1.0 μm step then began after a considerably shorter latency; such a step was always completed in two phases, each about 0.5 μm in amplitude, with an intervening notch that seemed to result from the inertial and resonant characteristics of the system. Movement velocities were measured accurately from records made with higher gain and expanded time scales. For a 1.0 μm step, the first and second phases had respective velocities of 0.94 and 0.69 μm/ms. Since the stepping motor proved not to reach full velocity on the first, step, higher movement velocities were attained

by giving preset bursts of steps at about 1500/s. Figure 9 shows that these high-frequency bursts became essentially fused into a single larger step, with a small pause always separating the total motion into two phases. With a 2.0 μm advance, the respective velocities of the first and second phases were 1.56 and 0.85 μm/ms, while a 3.0 μm advance gave respective velocities of 2.0 and 1.4 μm/ms. With high-frequency bursts of 4 or 5 steps, there was little further increase of movement velocity.

From the standpoint of penetrating cells, while also minimizing cell damage, three aspects of a small but rapid electrode advance are probably important. First, acceleration should be great, so that full velocity will be available over as much of the step as possible. Second, the velocity of advance should be as high as possible, to assist in cell penetration. Third, vibrations following the step should be minimal, since after-vibrations can damage the cell and reduce both the quality and duration of intracellular recordings. Figure 9 shows very high accelerations, full velocity being reached almost instantaneously for advancing steps of 1-3 μm. For the case of a 1.0 μm step, velocities of about 1.0 μm/ms have been reported for both electromagnetic and piezoelectric advancers (Fish et al., 1971; Tupper & Rikmenspoel, 1969). With a 1.0 μm step, the velocity of our first phase of movement was in that same range, while the velocity of the second phase was slightly less. With a 3.0 μm step, our velocities during both phases of movement were about double those attained during a 1.0 μm step. Figure 9 shows vibrations following a 1-3 μm advance to be quite small on both a relative and absolute basis, and these vibrations appear much less significant than those reported with electromagnetic devices (Fish et al., 1971; Tomita, 1965). In summary, for all three critical aspects of the advancing step, our results are similar to or better than the characteristics reported for previous devices that have been designed for high-speed electrode advances over short distances.
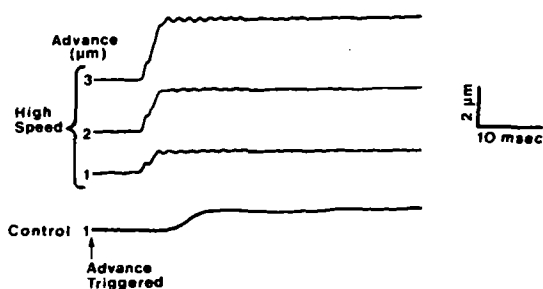

Fig. 9. Records of advancing movements by a standard Kopf stepping microdrive (control) and by the same hydraulic unit following high-speed modifications. All records made with a Unimeasure/80, using a bandpass of 0–10.000 Hz. For further description. see text.

Automatic stopping of electrode upon cell penetration

The electrode advance was programmed from a Nova 2/10 mini-computer, using the external control terminal of the Kopf control unit. The computer program specified the stepping rate and the total distance the electrode was to be advanced. If the stepping rate is too high, interactions between steps can result in undesirable vibrations. We used a rather low rate of 10 steps/s, but Fig. 9 suggests that rates up to about 40 steps/s could probably be used, and these higher rates might be useful for deep penetrations in the central nervous system. An A–D converter monitored the d.c. potential recorded by the advancing electrode, and whenever a negative d.c. shift occurred that exceeded a preset trigger level, the computer stopped the advance. Thus electrode advancement was initiated by pressing a button, and the electrode stopped automatically when a cell was penetrated whose membrane potential exceeded a selected trigger level. After recording from a given cell, the advancer could be restarted by an override signal. Upon reaching the preset total depth, the advancer was stopped by an internal signal. At that point another depth run could be initiated if desired, but the electrode was usually withdrawn. A high-speed return was provided to withdraw the electrode a preset distance at about 1000 steps/s.

While the described controls are quite convenient, the major advantages are to find cells quickly, while minimizing the damage resulting from penetrating a cell. More high-quality recordings may thus be obtained during a given experiment. With each step giving an axial advance of only 1 μm, and the advancer always stopping immediately after the step that penetrates a cell, the distance of membrane penetration is limited to about 1 μm and in some cases may be considerably less. The importance of controlling the depth of cell penetration is readily appreciated by examining the electrode taper. Though our puller provides electrodes that taper only slightly in the region of the tip, Fig. 4 shows that the diameter increases about 0.1 μm for each 1.0 μm of axial distance behind the very tip. For small cells this is a significant increase of diameter, so the advantages of ultrafine tips will be compromised unless the distance of penetration is severely limited.

In obtaining the described control of electrode advancement, a computer is convenient but not necessary. The main control functions could also be obtained with a relatively simple electronic unit featuring a voltage comparator.

TESTING OF INTRACELLULAR RECORDING TECHNIQUES IN SMALL CELLS

The capabilities of intracellular techniques can be assessed accurately only by testing them in a well-defined preparation. All of our techniques for intracel-lular work in small cells have thus been tested in the isolated, inverted, and perfused retina of the toad, *Bufo marinus*. Intracellular recording was concentrated in rod outer segments, which are shown by SEM in Fig. 10. The densely represented red rods have long cylindrical outer segments; the scarcer green rods have cylindrical outer segments of similar diameter but are shorter. Because of their size, Fig. 10 shows that the outer segments of the red and green rods are the only reasonable targets that are available for intracellular recording in this preparation until reaching the inner segments of the red rods. A total of 49 rod outer segments, obtained from toads of three different sizes, were measured in the fresh state immediately after being shaken off into toad Ringer solution on a microscope slide. The diameters ranged from 5.0 to 7.5 μm and averaged 5.9 μm.

As a preparation for testing microelectrode methods, the outer segments of toad rods have two major advantages. First, they provide a dense population of structures of uniform size. When penetrating this layer of the retina, there is no doubt about the sizes of cells penetrated, unlike the case of a tissue containing cells of various sizes. Second, they provide an especially difficult preparation for intracellular recording. In addition to their small size, they are also rather free to move, particularly at their tips, when contacted by an electrode. Hence microelectrode methods that are effective in this preparation should be equally or more effective in most other preparations.

Testing was done mainly with ultrafine single barrel micropipette electrodes formed from Omega Dot tubing with our special puller, then filled with 5 M K acetate, after which their resistances averaged about 70 MΩ. The retina was placed on the convex surface of a glass contact lens, which was mounted in a perfusion chamber on a microscope stage. The receptor surface was visualized with infrared illumination and a 40× water immersion objective, combined with a high-resolution television camera and monitor. Contact of the electrode tip with the receptors was usually seen accurately by movement of the tip of a single outer segment. At our angle of penetration, an advance of about 155 μm was then required along the electrode track before reaching the inner segments of the red rods.

During such penetrations intracellular recordings were readily obtained. Several successive recordings along a single penetration were not unusual, and as many as 7 outer segments have been recorded along a single electrode track. It has thus proved easy to make 30–40 intracellular recordings during a single experiment. More important, we obtained membrane potentials up to 46 mV and hyperpolarizing light responses up to 32 mV. These values are similar to the largest values that have been reported in a variety of vertebrate photoreceptors, though most previous work has been in larger and more firmly held structures, such as inner segments of *Necturus* and turtle.

The largest responses were usually the most stable, probably because both of these results depend upon penetrating the cell with minimal damage, and most cells with large responses have given stable recordings that were not lost spontaneously. In some cases neither the membrane potential nor light response has shown any detectable deterioration for 2–3 h. Hence we typically spend only a short time obtaining a high quality recording and perform an entire experiment on only one or two cells. This extreme stability permits experiments not previously possible, such as studies of dark adaptation in rod outer segments. It also permits a variety of observations to be made on the same cell, so that the results can be compared accurately.

As expected, there was a tendency for penetrations to be made more readily at deeper levels of outer segments, where these structures can move less freely when contacted by the electrode. Recordings from the outer tips, however, at the very point of contact or only a few μm beyond, sometimes yielded large and stable responses. For the tips of outer segments, it proved useful to advance by 3.0 μm steps, as illustrated in Fig. 9. Apparently the higher velocity and greater distance of the 3.0 μm advance was helpful for penetrating these small structures when they were especially free to move.

The reported results pertain to both beveled and unbeveled electrodes, but the beveled electrodes generally performed better. Apparently our ultrafine tips are sufficiently small that beveling was not critical for cell penetration but was still helpful. For injecting Procion yellow, however, beveling dramatically reduced problems of electrode plugging. This permitted current of at least 2.0 nA to be injected for 5 min or more without any significant spontaneous fluctuations.

The dual channel electrodes performed at least as well as the single channel electrodes, and perhaps even better, for readily obtaining high quality intracellular recordings. This was in spite of the dual channel electrodes having slightly larger tips. Hence the ease of cell penetration further indicates the presence of the theoretical spear on the dual channel tips. This theoretical spear has shown no indication of being especially fragile, since the low coupling resistance was sometimes maintained through several penetrations. Though all of our electrode tips are stiffened by having relatively short tapers, the thick septum must further stiffen the dual channel tips. This feature may likewise aid in penetrating cells, and it should be especially helpful in penetrating deeply, or through tough tissue, en route to target cells. Because of its advantages for cell and tissue penetration, the thick septum theta tubing also has promising applications as a single channel electrode. For this case no special treatment is required at the back end, contact being desired with both channels, which may thus be used in parallel as a single channel having half the resistance of an individual channel.

Of the numerous possibilities offered by dual channel electrodes, some sample applications have been tested in the outer segments of toad rods. Experimental voltage clamping has been done, and ions have been injected intracellularly through one channel while recording their effects on light responses with the other channel. Also, these electrodes have been used in both outer and inner segments of toad rods to make Procion yellow injections with one channel while 5 M K acetate was used in the other channel for low resistance recording. Finally, these electrodes have proved satisfactory for voltage clamping in rod inner segments in the retina of the snapping turtle (COPENHAGEN, OWEN & BROWN, 1976). Hence these electrodes may be used for a variety of dual channel experiments.

DISCUSSION

Since our test results were obtained in a quite difficult preparation for intracellular recording, it appears that the new techniques solve the main problems that have pertained to intracellular work in small cells, while also offering significant advantages in large cells. In the current context of neurophysiology, these techniques promise two especially broad advantages, the limits of which can be established only by future work. First, it seems highly advantageous that cell size should no longer be a crucial consideration in choosing an experimental preparation, so that this choice can be based more exclusively on considerations directly related to experimental purposes. Second, it is desirable for cells of all sizes that methods be available for conducting intracellular work with greater efficiency. This should improve the data base in many cases, save experimental time and animals, reduce the funds required for each project, and speed overall experimental progress.

It seems likely that these techniques will find particularly extensive applications in the vertebrate central nervous system, where the amount of intracellular work that will ultimately be required makes the need for experimental efficiency especially compelling. For example, it has been demonstrated in the retina that much signal processing is accomplished by relatively small cells generating only slow potentials, which can be studied in detail only by intracellular methods. If this principle holds generally in the central nervous system, as may be expected, intracellular recording is urgently required in small cells of the brain and spinal cord. Many other problems in the central nervous system, such as the detailed connectivity between cells, likewise require intracellular techniques.

The use of intracellular recording has thus far been severely limited in the vertebrate central nervous system, partly because most cells have been too small, and partly because of troublesome circulatory and respiratory pulsations. Methods of reducing the pulsations have long been available, and further reduc-

tion seems possible. Also, most cells in the central nervous system are significantly larger than the outer segments of toad rods, and more movement can probably be tolerated in those larger cells. Though seldom mentioned, a potentially very helpful factor is an apparent sealing process that occurs after a cell has been penetrated. In rod outer segments this has been revealed by a gradual increase in both membrane potential and light responses during the period just after penetrating a cell. Similar effects have been seen by other investigators and suggest a sealing of the membrane around the electrode tip, perhaps a molecular type of wound healing. The time required for such healing may be expected to decrease with smaller tips that cause less damage, and with our ultrafine tips the sealing process was usually complete in about 30 s. Following this sealing process the attachment between electrode tip and membrane was astonishingly strong. Sometimes the electrode could then be moved forward or backward by 20 μm, or even more, without losing the intracellular recording from an outer segment. These observations suggest

that if ultrafine tips can be inserted into brain cells for relatively short periods, the sealing process may occur rapidly and thereafter prevent the recording from being lost by small pulsations. Also, E. MAYERI (personal communication) has noted in large *Aplysia* cells that hyperpolarizing current increases the rate at which a cell's input resistance increases to a stable value just after penetration. This indicates that hyperpolarizing current may further speed the sealing process. In view of the experimental possibilities and the available techniques, it thus appears that the intensive application of intracellular recording to the vertebrate central nervous system is now a goal that is not only desirable, but also realistic.

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Micropipette puller design: form of the heating filament and effects of filament width on tip length and diameter

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Though the ultrafine short tips provided by our micropipette puller are helpful for many preparations, longer tips are still needed for some cases where target cells for intracellular work are deeply embedded in tissue. Because the original 'loop' type of heating filament proved unsuitable for widening to provide the longer tips, we studied simpler forms of heating filaments. By comparison with the common loop design, a rectangular trough filament proved to have many advantages without any significant disadvantage. In particular, this type of filament may be used at varying widths to provide long tips as well as short ones. Thus our micropipette puller can now provide tip lengths that extend upward by continuous gradations from about 6 to 27 mm, while maintaining tip diameter in the ultrafine range of 0.1 μm or less. These long tips have proved more reliable and free from bending than long tips traditionally provided by the Livingston puller. By using narrow filaments in conjunction with other parameters that influence tip size, tips have also been formed with diameters up to 1.5 μm and lengths of only 2–3 mm.

Introduction

It has usually been assumed that the heating filament of a micropipette puller would ideally be cylindrical to soften a glass capillary uniformly around its central axis. In the original design of our airjet micropipette puller, that assumption was followed by forming a flat ribbon of platinum–iridium into a loop (Brown and Flaming, 1977). With that heating filament the puller was shown to provide very reliable tips in the ultrafine range, as defined by outer tip diameters of 0.1 μm or less. These tips could also be much shorter than previously attained for such fine tips, as little as 6.0 mm when measured from the earliest discernible taper to the ultimate tip. In obtaining these short tips it was necessary to use a relatively narrow heating filament (2.0 mm). It also proved critical to cool the filament rapidly as the tip was formed.

Though the ultrafine short tips have proved advantageous in many preparations, long tips are still required in some cases where a considerable depth of nervous tissue must be penetrated before reaching the target cell. This is because the adhesion of nerve tissue to glass, combined with the rapid taper of a short tip, cause significant resistance to penetration. To date the most practical solution of that problem is a relatively long slender tip, similar to those provided by the well known Livingston puller (Livingston and Duggar, 1934). Hence one goal of this work was to increase the versatility of our puller by making it readily adaptable to form longer ultrafine tips. In so doing, it was anticipated that the advantage of high reliability could be extended to the longer tips.

An effective simple method for producing longer ultrafine tips is a widening of the heating filament. This requires that filaments of various widths be easily interchangeable, which proved inconvenient with loop filaments. More important, when the rather long band that forms the loop filament was widened appreciably, its power requirements exceeded the design of the power supply; also, the airjet system was insufficient to cool such filaments rapidly. As a result, the tips formed were inconsistent and unsatisfactory. In experimenting with other filament shapes we learned that the form of the heating filament can be simplified without any significant disadvantage and with a variety of advantages. These include the use of interchangeable heating filaments that can be widened for increasing the length of ultrafine tips or narrowed for increasing tip diameter while further reducing the tip length.

The experimental work of this report was conducted on our own design of micropipette puller, in part because research on micropipettes is more difficult and of less assured significance when conducted with pullers of low reliability. Since the heating element is a critical component of all micropipette pullers, it is believed that the general principles revealed in this work should apply similarly to other puller designs.

The loop filament

The 3 filament forms used in this work are shown in Fig. 1. All filaments were made from a sheet of 90% platinum and 10% iridium that was 0.002 in. thick.

In Fig. 1 the top drawing shows the 'loop' type of filament in our original puller design. Our 'standard' filament of this type has remained 2.0 mm in width, as was used in forming the micropipettes described with the puller design (Brown and Flaming, 1977). The width of this filament can be reduced to 1.5 mm to obtain even shorter tips, while still adequately shielding the tips from the airjet so that tip bending does not become a problem. The filament width can also be increased to about 2.5 mm without encountering the problems that occur with still wider filaments. At that filament width, tip lengths of about 15 mm may be obtained, but still longer tips are required to cover the desired range of applications. Because of its horizontal orientation the loop filament can sag at high filament temperatures if the

horizontal 'legs' of the loop are too long. That problem was solved by shortening the filament legs to 4.5 mm, as shown in Fig. 1.
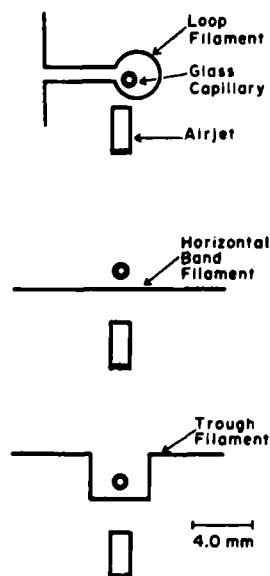

Fig. 1. Scale drawings of the 3 kinds of heating filaments used in this work.

While theory suggests that tip bending would be minimized by orienting the glass capillary tubing in the center of the loop filament, for uniform heating of the glass, this has proved not to be the case. Instead, tip bending is empirically minimized by placing the glass capillary as close as possible to the loop near its lower leg, as shown in Fig. 1. This suggests that uniform heating of the glass capillary is not as important as generally believed for forming straight tips. It also suggests that tip bending is strongly influenced by other factors that are minimized by the illustrated configuration.

In the original description of our puller, the air tube leading from a small micrometer-controlled orifice was divided at a Y-junction to form two airjets, which were mounted symmetrically above and below the loop of the heating filament. When we subsequently removed the heating filament and held a strip of paper between the two airjets, puffs of air were noted to deflect the paper randomly in one direction or the other. So the air in any given puff was passing mainly through one channel or the other, rather than being divided about equally between the two channels. Upon consulting with airflow engineers, it was learned that this result may be expected when an orifice limiting the airflow precedes a Y-junction. Though the random variation of effectiveness between the two airjets had no obvious consequences for the micropipette tips, it may slightly reduce the reliability of tip characteristics. In any event, since each puff of air was passing primarily through only one airjet, a second one appeared unnecessary. We thus kept only the lower

airjet and this has proved satisfactory. As shown in Fig. 1, this airjet is located near the part of the loop that delivers most of the heat to the glass capillary. Other portions of the loop deliver less heat to the glass; though they are also farther from the airjet, they appear to be cooled adequately by heat conduction through the filament to the site cooled most directly by the airjet.

The horizontal band filament

In studying the feasibility of simpler filament forms, we looked first at the ultimate simplicity of a straight horizontal band, as shown in the middle drawing of Fig. 1. This band was placed about 0.5 mm below the glass capillary, rather than over it, to take advantage of convective heat rise and thus improve the efficiency of heat delivery to the glass capillary. With this type of filament the location of the airjet proved less critical, and it was mounted about 2.0 mm below the center of the horizontal band, where it could efficiently cool that portion of the filament delivering the most heat to the glass capillary. There is some advantage in having a reasonably large separation of the airjet from the filament, since this allows the puff of air to cover better the portion of the filament to be cooled; also, the lesser velocity of resulting air currents in the vicinity of the forming micropipette may reduce problems of tip bending.

Micropipettes formed from this type of heating filament showed no significant tip bending, even when the filament was widened to 5.0 mm to form tips as long as about 23 mm. In fact, this form of heating filament gave less difficulty with tip bending than the loop filament. It appears that several factors contribute to understanding this result. First, asymmetric heating of the glass capillary is probably significant only while the capillary diameter remains fairly large. When the capillary diameter has become quite small, as it has following the rapid initial taper, the temperature gradient between the lower and upper sides of the tubing apparently becomes too small to contribute to tip bending. Second, the positioning of the glass capillary to prevent tip bending is much more critical with the loop filament than with the horizontal bend. This suggests that complex patterns of convective airflow, which must occur within the loop, may contribute to tip bending. By contrast, with the horizontal band filament the convective airflow is only upward and thus less complex. Third, if the glass capillary is centered in a loop filament, tip bending is a consistent problem when tips are formed normally by use of the airjet. If the airjet is inactivated, the normal length of the tip is quite straight, but an extremely long slender filament extends well beyond the normal tip length and makes the micropipette unusable. These observations indicate: (1) that a minimum amount of air cooling is required to form usable tips; and (2) that under certain conditions the airjet contributes significantly to tip bending. This aspect of tip bending may also be less severe with the horizontal band filament because of its simpler form and the less complex resulting air currents. Fourth, it is often considered that a horizontally oriented micropipette puller will give gravity-bent tips, especially if they are relatively long. In our puller the fast pull is completed within 5-8 ms (Brown and

Flaming, 1977). This is probably too little time for the tip to be bent significantly downward by gravity. Alternatively, this effect may be present but almost exactly cancelled by other factors, such as upward air currents resulting from convection and the airjet.

The trough filament

Compared with the loop filament, the horizontal band filament delivers considerably less heat to the glass capillary. Thus the horizontal band is not satisfactory for borosilicate (Pyrex) tubing that is thick-walled or large in diameter, nor is it satisfactory for aluminosilicate tubing. The horizontal band filament also bends either upward or downward as the filament lengthens during heating; this alters the distance between the filament and the glass capillary, which is a critical variable for pulling micropipettes. Both problems are overcome by the rectangular 'trough' filament shown in the lower portion of Fig. 1. The bottom of the trough functions much like the horizontal band filament, but the sides of the trough filament contribute to the total heat delivered. Also, the sides of the trough filament can tilt slightly as the filament lengthens during heating, thus taking up most of this effect. While a slight downward movement of the trough filament may still occur during heating, this should be consistent in both direction and amount and should not reduce the reliability of the micropipettes that are formed. In adapting our micropipette puller for horizontal band and trough filaments, it was only necessary to redesign the filament mount; this was done with special attention to convenience in changing heating filaments. When using double-barrelled micropipettes based upon two capillary tubes fused together, the two tubes should be equidistant from the bottom of the trough to avoid tip bending. Provision has also been made for this special case by modifying the tubing clamps on the pipette carriers.

After some experimentation, the dimensions chosen for the trough filament were 4.0 mm for the length of the bottom segment, 3.0 mm for the height of the sides, and 3.0 mm for the filament width. These values suffice for most purposes and hence provide a satisfactory standard, from which alterations may be made for special purposes. In evaluating this type of filament we have used primarily borosilicate tubing with outer and inner diameters of 1.0 and 0.5 mm, respectively, a solid fiber 0.1 mm in diameter being fused along the entire length of the inner bore for convenient filling of micropipette tips. We refer to this as standard tubing, since it is the type most commonly used in neurophysiology. One reason for choosing the 3.0 mm width for the trough filament was that it proved to form very similar tips from standard tubing as were provided by the loop filament at a width of 2.0 mm. Also, the heat delivery from this trough filament has proved adequate for all types of glass that are now commonly used in neurophysiology. This includes borosilicate tubing up to at least 2.0 mm in outer diameter and 0.5 mm in wall thickness. It also includes aluminosilicate tubing with outer and inner diameters of 1.0 and 0.75 mm, respectively, the only dimensions in which this type of tubing is thus far readily available. With such heavy borosilicate tubing, or aluminosilicate tubing, trough

filaments appreciably narrower than 3.0 mm deliver too little heat to form tips. But with these tubings wider trough filaments, up to 6.0 mm, are useful if longer tips are desired. With standard tubing the width of the trough filament may be varied from 1.5 to 6.0 mm, for the control of tip length, without encountering any special problems. Even at the width of 6.0 mm, the power supply has proved adequate to heat this filament to the burnout point, and tip length may be modulated adequately by the airjet system. These features result from the trough filament having a total length of only 12 mm between the binding posts, compared to 18 mm for the loop filament. With the trough filament we have not noted any significant problem of tip bending, even at the maximum filament width of 6.0 mm. It thus appears that the trough filament combines the advantages of the horizontal band and loop filaments.

Evaluation of trough filament

Comparison with loop filament

When compared with the loop filament, the trough filament offers the following advantages:

(1) The trough filaments are easier to form.

(2) The trough filaments are easier to mount in the puller and are then more easily interchanged to vary filament width.

(3) The trough filament is less vulnerable to being struck when positioning the glass capillary because there is no need to thread the glass capillary through a loop.

(4) The trough filament requires less power because it is shorter than the loop filament, thus offering less electrical resistance and requiring less voltage to attain a given filament temperature.

(5) The trough filament requires a lesser airflow to provide the minimum cooling needed to form usable tips. This is partly because the trough filament is shorter; also, upper portions of the loop filament that contribute to heating the glass, but are rather inefficiently cooled, have been eliminated. Because of the lower minimum airflow that is required by the trough filament, the range of airflow available to control tip length is increased.

(6) As a consequence of items 2, 4 and 5 above, the trough filament may be greatly widened to form longer tips, as required for certain preparations.

By contrast, we have not identified any significant disadvantage of the trough filament by comparison with the loop. Though the total heat delivered to a glass capillary by the trough filament is somewhat less than that delivered by a loop filament of the same width, this does not appear significant since all tubings commonly used in neurophysiology are readily handled by either filament. In any special cases requiring even more heat delivery, the 'standard' trough could readily be modified by lengthening its sides or bringing them closer together.

Measurement of tip diameters and lengths

Fig. 2 shows the effects of varying filament width upon both tip length and tip diameter. In obtaining these results, the width of the trough filament was varied while keeping all other pulling variables as constant as possible. For each filament width, a filament was burned out and the reading on the digital scale of relative filament current at the burnout point was noted. These burnouts did not occur at bends in the filament but were consistently at the center of the filament just above the airjet. Though the heating current must be quite uniform along the filament, between the two binding posts, the color temperature of the filament is always highest in the center where the burnout occurs. This is undoubtedly because the heavy metal binding posts act as heat sinks that draw off heat from both ends of the filament. For filaments 3.0–6.0 mm in width the filament current was then reduced slightly below the burnout point, a reduction of 10 units on the digital readout, and that value was used to form all micropipettes with a given filament width. The burnout temperature may be assumed quite similar for filaments of different width, and when filament widths of 3.0–6.0 mm were used at a constant airflow setting, filament current could be reduced over a considerable range below the burnout point without significantly affecting either tip size or tip length. For filaments narrower than 3.0 mm, however, tip size increased sharply as filament current was reduced. Hence for these narrower filaments the filament current was reduced only very slightly below the burnout point, an attempt being made to match the color temperature at the center of the filament with the color temperature of the wider filaments. These procedures provided near-maximum filament temperatures that remained approximately constant with varying filament width and had little or no effect upon tip measurements.

The length of the slow pull was always 0.50 in. The strength of the slow pull on each micropipette carrier was determined by an 80 g weight suspended from a flexible cable, which passed over a pulley and was thence attached to the carrier. The digital readout for strength of the fast pull was always set at 1250, which provided 50 V across the pulling solenoid. For the airjet system the pressure was 50 psi. Of course the minimum airflow needed to produce usable tips increases with filament width. The micrometer control of the needle valve was thus set to provide the minimum airflow required for usable tips with the 6.0 mm filament, and that airflow setting was used for all filament widths. The actual reading of the airflow micrometer was 0.075 in. But the reading to obtain the same airflow might be somewhat different on another micropipette puller of our design. This is because the zero point on the airflow micrometer does not provide an identical airflow on all instruments.

Standard glass tubing was used in this work. As with the horizontal band filament, the glass tubing was located about 0.5 mm above the bottom of the trough filament, and the airjet aperture was about 2.0 mm below the filament, as shown in Fig. 1.

We have now developed a convenient and reliable method for mounting micropipette tips for high resolution scanning electron microscopy (SEM), as described in a separate paper in preparation, and that mounting method was used in this work.

Prior to SEM observations the tips were coated with 10 nm of gold. This has proved very close to the minimum thickness of gold that suffices for these purposes, and it is somewhat less than the 15 nm used previously (Brown and Flaming, 1977). Photographs illustrating the resolution that we attain with ultrafine tips at a magnification of about 40,000 × have already been provided (Brown and Flaming, 1977). Tip diameters were measured from such photographs, and twice the thickness of the gold coating was subtracted to obtain accurate diameters for the glass tips. Following our previous practice, tip length was measured from the very beginning of the taper to the ultimate tip.

Filaments were used at 6 different widths, which cover the usable range of filament widths under the conditions described. On any given day of SEM observations, all micropipettes were formed at a given filament width and then measured that same day. The number of tips successfully measured in these daily filament-width groups varied from 12 to 15 with an average of 14.4. The order of filament widths examined was as follows: 1.5, 3.0, 5.2, 2.25, 4.0, 6.0 and 3.0 mm. The final retest at the 3.0 mm filament width was to determine whether any change had occurred between the initial test and the retest of that filament. The respective test and retest values were 12.6 and 12.0 mm for tip length and 0.079 and 0.073 μm for tip diameter. These differences between test and retest values are too small to require consideration in the interpretation of our results.

Fig. 2 shows that when the 6.0 mm filament was used with minimal air cooling, the tip lengths averaged 26.8 mm. In our experience ultrafine tips cannot be made shorter than about 25 mm on the Livingston puller. Hence our range of tip length now extends to somewhat above the shortest ultrafine tips provided by the Livingston puller. As filament width was reduced, tip length decreased progressively and markedly. As this occurred, tip diameter remained quite constant at about 0.08 μm down to a filament width of 3.0 mm, which gave a tip length of 13.0 mm. This finding supplements earlier evidence that over a considerable range of tip length, our puller can provide ultrafine tips of quite constant size (Brown and Flaming, 1977). Below a filament width of 3.0 mm, Fig. 2 shows that tip length continued to decrease rapidly, while tip diameter began to increase. A smooth curve fitted by eye to the data on tip diameter suggests that tip diameter did not increase to any statistically significant extent until tip length had fallen to about 10.0 mm. In any event, at a filament width of 2.25 mm the tip length had fallen to 7.9 mm and the tip diameter had risen only to 0.1 μm, which is still within the ultrafine range. Our definition of the ultrafine range of tip sizes is operationally useful because we find that tips in this range function extremely well for intracellular work in the outer and inner segments of toad rods, which have average diameters of only about 6.0 μm (Brown and Flaming, 1977). At a filament width of 1.5 mm, Fig. 2 shows that tip length had fallen further to 5.4 mm but tip diameter had risen sharply to 0.18 μm. That rise of tip diameter is quite significant, at least for certain preparations of small cells. In the outer and inner segments of toad rods, for example, we have found that the frequency of cell penetration with 0.18 μm tips drops to about one-third of that obtained with ultrafine tips.

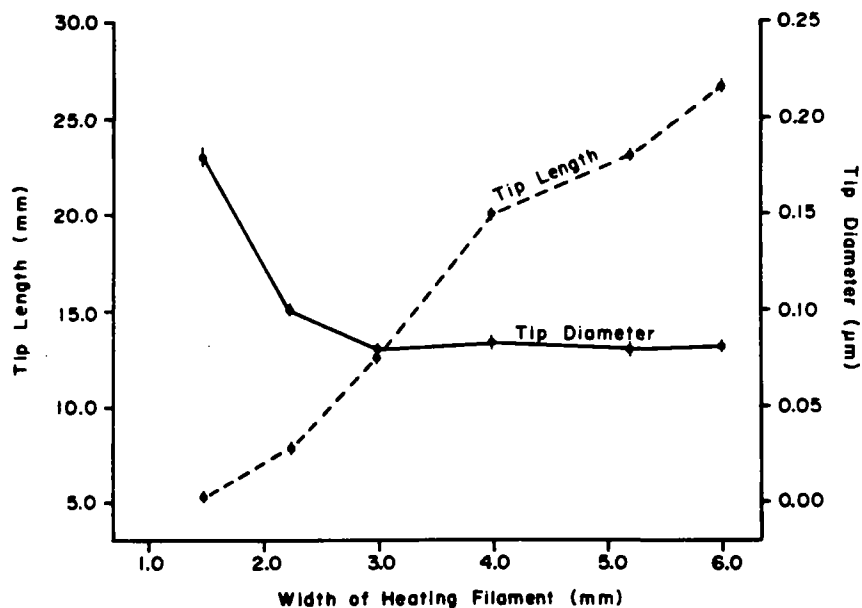The results shown in Fig. 2 are not inconsistent with our earlier report that

Fig. 2. Tip length and tip diameter as a function of width of the trough type of heating filament. Vertical error bars indicate ± 1 S.E. of the mean. For further details, see text.

ultrafine tips could be obtained down to tip lengths of 6.0 mm (Brown and Flaming, 1977). This is because tip length was reduced in Fig. 2 by decreasing filament width while holding airflow constant. When the filament becomes quite narrow, the total heat delivery becomes inadequate to heat the glass sufficiently to form ultrafine tips. Hence when tip length is reduced by decreasing filament width, a point is inevitably reached beyond which a further reduction of tip length is accompanied by an increase of tip diameter, as Fig. 2 illustrates. By contrast, our earlier results were obtained with a loop filament of fixed width, the tips being shortened by increasing airflow. In that case the glass was always thoroughly heated at the beginning of tip formation, only the rate of cooling of the tip being modulated by the airflow, and that procedure appears preferable for producing ultrafine tips of minimum length.

In certain applications such as patch clamping, it is desired to have outer tip diameters up to about 1.0 μm to provide inner tip diameters of about 0.5 μm (Sigworth and Neher, 1980). Fig. 2 indicates that narrow filaments can be useful toward that goal, while having the additional advantage of producing particularly short tips. We have also determined that thin-walled tubing increases tip diameter, as theoretically predicted (Brown and Flaming, in preparation). By using narrow filaments and thin-walled tubing, with respective outer and inner diameters of 1.0 and 0.75 mm, we have recently produced tips only 2–3 mm long with tip diameters up to about 1.5 μm. Under given pulling conditions the tip sizes are consistent, a result that appears not to have been achieved previously for such large tips. This may solve the problem of consistently forming tips of appropriate sizes for patch

clamping. Since these tips are formed in the puller, rather than being pulled to a small diameter and then broken, it is also possible that the additional step of fire polishing may be eliminated. Hence these tips must now be evaluated in patch clamping applications. Experiments are likewise being conducted to study the interacting effects of all the critical variables in micropipette tip formation with respect to both tip length and tip diameter.

Applications of short and long ultrafine tips

The importance of varying tip length for different requirements is well illustrated by the intracellular retinal work of our research group. The ultrafine short tips have proved valuable in the isolated and inverted toad retina, where intracellular recordings have been made from the outer and inner segments of rod photoreceptors (Brown and Flaming, 1977). In that work the approach has been directly to the exposed tips of the outer segments. Because high quality and extremely stable intracellular recordings have been readily attained, some findings have been obtained that would otherwise have been very difficult and time consuming or not feasible at all (Brown and Flaming, 1978; Flaming and Brown, 1979). Similar advantages of the ultrafine short tips have been reported to us from many other investigators. In another project of our research group, intracellular work has been conducted in the isolated and perfused eyecup preparation of the Eastern grey squirrel (Charlton et al., 1981). The retinal target cells in this work have been primarily horizontal cells and photoreceptors, which in this preparation must be approached from the vitreous humor. Though the retina itself is only about 200 μm in thickness, there is always an overlying layer of vitreous humor that is quite viscous and variable in depth. In order to reach the target cells through the overlying tissues, it has proved necessary to use longer tips than provided by the original design of our puller.

Comparison of our long tips with those formed by Livingston puller

For intracellular work in the squirrel retina, relatively long micropipette electrode tips have been formed both by wide trough filaments in our puller and by the Livingston puller. The former group of microelectrodes offered several advantages. First, tip length could be varied gradually up to about 27 mm, and tips considerably shorter than the maximum could often be used in this preparation, but we have not been able to obtain ultrafine Livingston tips shorter than about 25. mm. Second, these electrodes performed consistently well in this preparation, indicating uniformity of tip size and tip configuration. By contrast, the Livingston tips were quite variable in performance, and valuable experimental time was often required to identify a useful microelectrode. Since most biological preparations can be used for only quite limited periods of time, the reliability of micropipette performance is often critically important. Third, there was no significant tip bending. By comparison, the Livingston tips showed tip bending, which was sometimes considerable and troublesome; though this may be avoided at low pull tensions, it occurred consistently at the high pull tensions required to form ultrafine tips.

Discussion

The requirement of rapid tip cooling in all micropipette pullers

As already mentioned, experience with our puller indicates that it is not possible to form a usable micropipette tip close to a heating filament unless the filament has been deliberately cooled. In our instrument two pipette carriers are pulled apart symmetrically to form two essentially identical micropipettes, the tips being formed as they separate at about the midpoint of the filament width. Though the tips are then pulled away from the filament very rapidly, inactivation of the airjet system results in tips that are so long, slender, and lacking in stiffness that they cannot be used. Thus, a minimum amount of air cooling is required to form usable tips, while still greater amounts of air cooling can be used to shorten the tips. The Livingston puller also makes two symmetrical micropipettes and solves the minimum cooling requirement by lifting the capillary tubing up and out of the heating filament as the tip is formed. So this upward motion of the glass capillary is an intrinsic aspect of the design, rather than an incidental effect of the pulling motion, and it is the most obvious probable cause of tip bending in that puller. In single-sided micropipette pullers, which have only one movable carrier, the tips are cooled by being formed well outside the heating filament in the direction of the movable carrier. A minor limitation is that the two tips are quite different in length, so for most work only one micropipette can be used from each pull. A greater limitation of single-sided pullers, which also applies to the Livingston case, is that cooling effects cannot be modulated to vary tip length.

Application of findings to other micropipette pullers

It is noteworthy that the Livingston puller has always employed a trough filament, but it is so deep that the sides provide relatively more heat than in the design we employ. Thus the Livingston puller must provide more uniform heating of the glass tubing, and in that respect it is probably intermediate between our trough filament and a loop filament. It is also noteworthy that several investigators found in the early 1960s that widening the filament of the Livingston puller produced sufficiently fine tips for intracellular work in vertebrate photoreceptors, and this finding was critical to initiating intracellular work in those small cells. Presumably that observation used the principle illustrated in Fig. 2 of this paper, which shows that if one starts from a sufficiently narrow filament, widening the filament can reduce the tip size while also increasing the tip length. In any event, the general principles found in this work may be expected to apply similarly to other puller designs. Thus, some other horizontal pullers could probably be improved significantly by the rectangular trough filament. And the general effects of filament width upon tip size and tip length may be expected to apply in all micropipette pullers, whether trough or loop filaments are used in either horizontal or vertical orientations, though the quantitative details of the effects undoubtedly vary between puller designs.

Acknowledgements

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