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Attempts to Measure Plasmalemma and Tonoplast Electropotentials in Small Cells of the Moss *Mnium* using Centrifugation Techniques

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Summary. Centrifugation techniques are described which allow that glass microelectrodes can be inserted separately into cytoplasm and vacuoles of intact cells of the moss *Mnium cuspidatum*. This provides unequivocal measurements of the plasmalemma potential (E_{co} between cytoplasm and outer medium). For various reasons, which are discussed in detail, determinations of the tonoplast potential (E_{vc} between vacuole and cytoplasm) remain ambiguous. In the moss cells equilibrated with artificial pond water (APW), E_{co} is highly negative (i.e. -130 to -190 mV). E_{vc} is either close to 0 mV or positive ($+60$ to $+65$ mV) depending on the interpretation of the measurements. However, there is no reason to suggest that under these conditions highly negative potentials occur at the tonoplast.

Plant cells are compartmented into cytoplasm and vacuole. The plasmalemma and tonoplast membranes in series are separating a frequently very thin layer of cytoplasm (c) from the aqueous media of the external solution (o) and of the vacuolar sap (v). Hence, a glass microelectrode with the tip in the interior of a cell will often record a potential difference (E_{cell}) which is equal to the overall potential between the vacuole and the outside (E_{vo}), and which is composed of the plasmalemma and tonoplast potentials (E_{co} and E_{vc}, respectively), so that

$$E_{\mathrm{cell}} = E_{vo} = E_{co} + E_{vc}. \tag{1}$$

However, it is not unequivocal that an electrode with its tip apparently in the center of a cell indeed measures E_{vo} (i.e., that $E_{\mathrm{cell}} = E_{vo}$). It may be argued that the tip is covered with some cytoplasm and tonoplast, with the tonoplast forming a fingerlike invagination, so that in fact $E_{\mathrm{cell}} = E_{co}$. In

an attempt to obtain undebatable measurements of E_{co} and perhaps also of E_{vo} in small cells, centrifugation techniques allowing to distinguish between cytoplasm and vacuoles have been used in the present study.

Materials and Methods

General

The moss *Mnium cuspidatum* was obtained from forests surrounding Darmstadt, Germany. The leaflets of the gametophytes were used soon after collection of the moss or after cultivation in petri dishes near a laboratory window for up to 9 weeks, where the plants were kept moist using tap water and vigorously grew new branches. Before use in the experiments, small branches were floated for 72 hr in aerated APW (0.1 mM KCl, 1 mM NaCl, 0.05 mM $CaSO_4$) at room temperature, so that the cells were in flux equilibrium with the external medium.

Glass microelectrodes were prepared using a Getra electrode puller and filled with 3 M KCl under reduced pressure in a desiccator. The external tip diameter of the electrodes was <0.5 µm. The tip potential and the electrode resistance were measured in APW before and after the cell potential recordings. Electrodes with tip potentials between 0 and -25 mV and resistances between 10 and 20 MΩ were selected for the experiments. Measurements were discarded if tip potentials and electrode resistances varied greatly before and after punching a cell. (The tip potential was substracted from the measured potentials.)

Moss leaflets were mounted in a small perspex chamber (2 cm^3) on the stage of a microscope. The chamber was flushed with APW at 0.2 $cm^3 \times min^{-1}$. The reference electrode was placed in the APW at the outlet of the chamber. The tips of glass electrodes were inserted into cells under 125 × magnification of the microscope (10:1, a 0.25 objective with 5.7 mm working distance) using Leitz micromanipulators. Recordings were made with a Keithley differential electrometer Model 604 and a Linseis pen recorder.

Centrifugation

To enable clear insertions of the electrode tip into the cytoplasm and into the vacuole, respectively, the cells were centrifuged. Small moss branches with the leaflets still attached were fastened in centrifuge tubes containing APW with the tips of the branches pointing towards the bottom of the tubes. Centrifugation was performed with a small laboratory centrifuge for 30 min at 1.6×10^3 *g* and at room temperature or for 30 min with a Christ ultracentrifuge at 20×10^3 *g* and at 0 °C. Centrifuged cells are shown in Figs. 1*a*, 1*c*, and 3. Centrifugation leads to accumulation of the cytoplasm with organelles in one part of the cells. As can be seen in Fig. 3, after ultracentrifugation a stratification of the cytoplasm is often obtained with the larger organelles (e.g., chloroplasts) sedimented further down than the rest of the cytoplasm.

Centrifugation of cells and tissues has been used earlier to get higher resolution of the cytoplasm from the rest of the cell in microscopic studies (Läuchli & Lüttge, 1968) and to resolve physiological activities of the cytoplasm (e.g., Goldsmith & Ray, 1973). Nevertheless, it appeared necessary to demonstrate by a number of controls that the separation of cytoplasm and vacuole after centrifugation did not severely damage the cells:

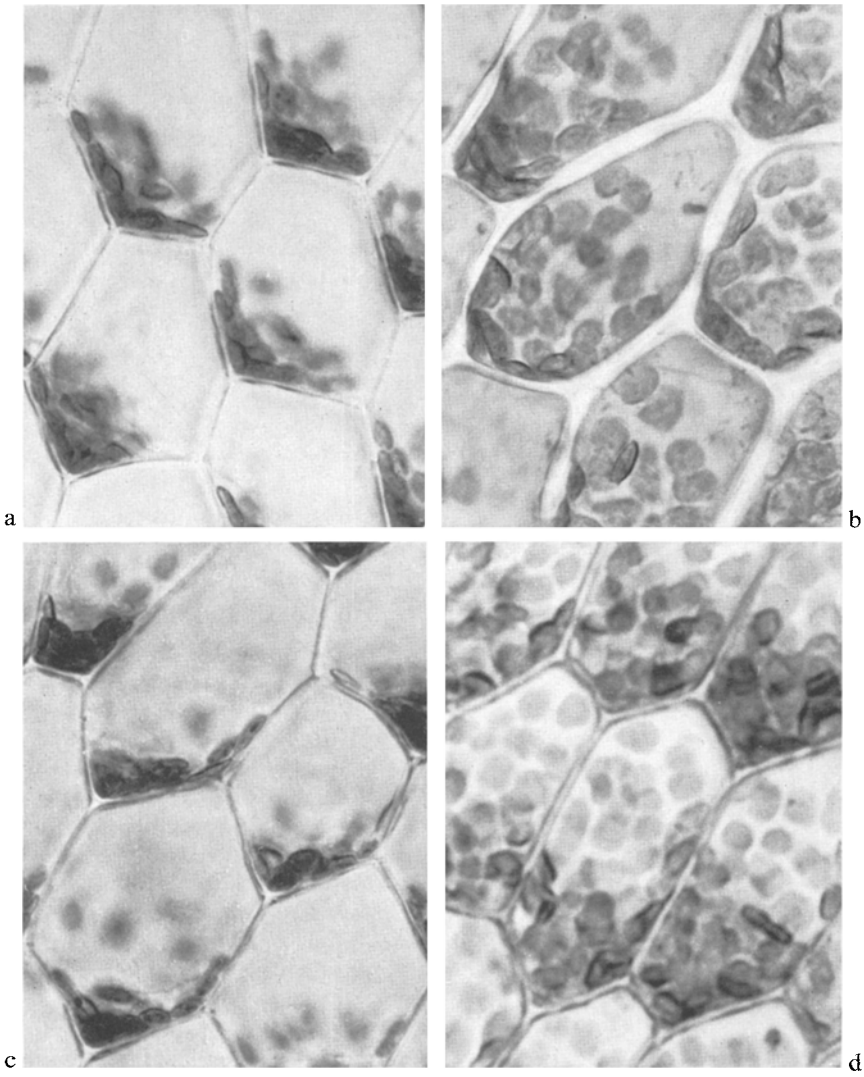

Fig. 1. *Mnium* leaf cells immediately after (*a*, *c*) and 3 hr after (*b*, *d*) centrifugation. *a*, *b*: $1.6 \times 10^3\,g$; *c*, *d*: $20 \times 10^3\,g$. Magnification 700 ×

(*i*) Läuchli and Lüttge (1968) observed that after ultracentrifugation, cytoplasmic streaming rapidly (i.e. within approx. 15 min) redistributed the cytoplasm and organelles in onion scale cells (*Allium cepa*), so that the cells appeared similar to noncentrifuged ones. Fig. 1 shows that a similar redistribution, although slower (fortunately for the measurements), occurs in the *Mnium* cells both after $1.6 \times 10^3\,g$ and $20 \times 10^3\,g$ centrifugation.

(*ii*) The stain neutral red, which is heavily accumulated in the vacuoles of some of the moss leaf cells is retained there after centrifugation.

Table 1. Potassium content and Nernst-potentials[a] of centrifuged and noncentrifuged moss material

Experiment		Soon after collection		7 Weeks cultivation	
		$K^+/\mu moles \cdot g_{FW}^{-1}$	E_{K^+}/mV	$K^+/\mu moles \cdot g_{FW}^{-1}$	E_{K^+}/mV
No equilibration in APW		75	–	–	–
72 hr Equilibration in APW		78	−172	67	−167
Time after centrifugation at $1.6 \times 10^3 g$:	0 hr	70	−168	67	−167
	1 hr	67	−168	70	−168
	2 hr	72	−169	76	−171
	3 hr	73	−169	79	−172
Time after centrifugation at $20 \times 10^3 g$:	0 hr	72	−169	77	−171
	1 hr	71	−169	80	−172
	2 hr	73	−170	83	−173
	3 hr	74	−170	91	−174

[a] $E_{K^+} = -\frac{RT}{F} \ln \frac{[K^+]_{tissue}}{[K^+]_{APW}}$; Eq. (2).

(*iii*) Potassium contents of moss branches were measured in acid extracts by flame photometry as shown in Table 1. No significant changes in K^+ levels were observed between noncentrifuged cells, centrifuged cells and cells recovering from centrifugation (*cf. also* Fig. 1). It should also be noted that equilibration with APW does not increase the K^+ levels, and that cultivation in the laboratory also has little effect.

(*iv*) Membrane potentials (E_{cell}) obtained with noncentrifuged cells and with cells 3 hr after centrifugation (*see* Fig. 1) were not significantly different, i.e., noncentrifuged cells after 72 hr equilibration in APW: -113 ± 3 mV (21 determinations), and cells 3 hr after centrifugation (data for $20 \times 10^3 g$ and $1.6 \times 10^3 g$ pooled): -117 ± 2 mV (11 determinations). Since ultracentrifugation involved cooling to 0 °C, the potential of noncentrifuged cells was also measured in APW of room temperature after a 30-min period at 0 °C. However, the potential was not affected by the cold pretreatment (i.e., E_{cell} was -117 ± 5 mV, 9 determinations). The more negative potassium Nernst-potentials (E_{K^+}) as compared with the measured potentials (E_{cell}) confirm an earlier suggestion that K^+ in *Mnium* might be taken up passively and exported actively and this shows that the material used here largely behaves as expected for *Mnium* (Lüttge, Higinbotham & Pallaghy, 1972).

The controls described above (*i*–*iv*) show clearly that centrifugation at both speeds used does not seriously damage cell integrity.

Abbreviations and Symbols

APW: artificial pond water

p.d.: electro-potential difference

E_{cell}: p.d. obtained with the tip of the electrode in a noncentrifuged cell

E_{co}: p.d. at the plasmalemma, i.e. between cytoplasm (c) and external solution (o)

E_{vo}^{true}: p.d. between vacuole (v) and external solution (o) of an intact normal plant cell with tonoplast and plasmalemma in series

E_{vo}^{app}: p.d. obtained with a centrifuged cell, where the tip of the electrode is *apparently* in the vacuole

E_{vc}: p.d. at the tonoplast, i.e. between vacuole (v) and cytoplasm (c)

By convention the algebraic signs of all potentials are referring to a zero potential of the respective *outer* compartment. Errors given are standard errors.

Results and Discussion

Fig. 2 shows membrane electropotential (p.d.) recordings obtained with the electrode tip in noncentrifuged cells, and in the cytoplasm and vacuole, respectively, of centrifuged cells. All measurements obtained during this study are summarized in Table 2.

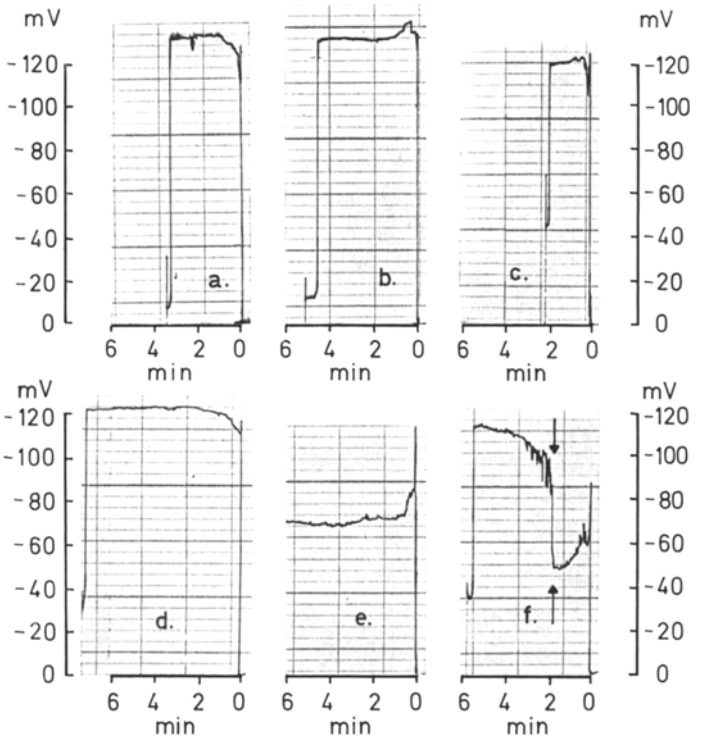

Fig. 2. Electropotential difference (p.d.) recordings. a: noncentrifuged cell; b, c: centrifuged at $1.6 \times 10^3\,g$; d–f: centrifuged at $20 \times 10^3\,g$. Position of the electrode tip: a: in the cell (measuring E_{cell}); b, d: in the cytoplasm (measuring E_{co}); c, e, f: apparently in the vacuole (measuring E_{vo}^{app}). The electrodes were inserted at time 0 (time scale running from right to left). The arrows in f indicate the time when the tip of the electrode became covered by migrating cytoplasm

Table 2. Potential differences measured with noncentrifuged cells, and with the tip of the electrode in the cytoplasm and in the vacuole, respectively, of cells centrifuged at $1.6 \times 10^3\ g$ or $20 \times 10^3\ g$

Time of cultivation in the laboratory (weeks)	Potential difference (p.d.) [mV]				
	E_{cell} (no centrifugation)	E_{co} ($1.6 \times 10^3\ g$)	E_{co} ($20 \times 10^3\ g$)	E_{vo}^{app} ($1.6 \times 10^3\ g$)	E_{vo}^{app} ($20 \times 10^3\ g$)
1	-177 ± 5 (7)	-192 ± 2 (5)	-176 ± 6 (2)		
2 to 6	-143 ± 14 (2)	-128 ± 2 (11)	-130 ± 6 (16)	-127 ± 8 (18)	-66 ± 5 (12)
7 to 9	-113 ± 3 (21)		-124 ± 2 (15)		-57 ± 3 (11)

For symbols *see* Materials and Methods. (The cells cultivated in the laboratory tend to have lower potentials than those used more or less directly after collection. This effect, which is not further discussed here, may have something to do with growth of new branches; *cf.* Lüttge and Bauer, 1968, and Lüttge and Krapf, 1968.)

We believe it is unequivocal that the tip of the electrode in the cytoplasm of centrifuged cells truely records the p.d. between the cytoplasm (c) and the medium (o) (i.e. E_{co}). The E_{co} values obtained are independent of the conditions of centrifugation (i.e. 1.6×10^3 or $20 \times 10^3\ g$; Table 2). Occasionally, the p.d. appeared to be slightly hyperpolarized by chloroplasts approaching the electrode tip, but this observation could not be supported statistically. We are confident that the electrode tip was not within chloroplasts in these measurements.

The measurements with the tip of the electrode *apparently* in the vacuole of centrifuged cells (E_{vo}^{app}) are much more difficult to interpret than those with the electrode in the centrifuged cytoplasm. First of all, the results obtained are very different after mild ($1.6 \times 10^3\ g$) and after strong ($20 \times 10^3\ g$) centrifugation, respectively (Table 2). Electron-micrographs (by courtesy of Dr. D. Kramer of our department) of cells centrifuged at $1.6 \times 10^{-3}\ g$ still show a thin layer of cytoplasm along the cell walls separating plasmalemma and tonoplast by about 0.1 or 0.2 μm. Thus, as discussed for E_{cell} in the introduction, it is debatable whether in mildly centrifuged cells E_{vo}^{app} reflects real E_{vo} (E_{vo}^{real}), or whether it reflects E_{co}, the tip of the electrode being covered by some cytoplasm and the tonoplast invaginating into the vacuole. Unfortunately, attempts to obtain electron-micrographs of ultracentrifuged cells have not been successful as yet. It is possible that in this case the plasmalemma and the tonoplast are very close together forming something like the "tight junctions" known to occur in certain situations between the plasma membranes of adjacent animal cells. Alter-

natively, the cytoplasm and the vacuole might be separated to such an extent that the tonoplast is no longer surrounded by cytoplasm. However, these alternatives can, perhaps, be discarded because under these conditions one would expect significant redistribution of ions (e.g. K^+) which was not observed (Table 1). Nevertheless, alterations of cytological structure by ultracentrifugation are possibly responsible for the great difficulties in inserting electrodes into vacuoles after ultracentrifugation. The potential often breaks down very rapidly and the cells appear destroyed by insertion of the electrode. On the other hand, in a fair number of insertions the p.d. is steady over many minutes at a value of approximately -60 mV (*see* Table 2 and Fig. 2*e*). In other cases the electrode records a low potential (~ -50 to -60 mV) for about 2 min, which then suddenly rises to a higher value corresponding to that of E_{co} (Fig. 2*f*). This lack of stability of the potential may be due to a poor and variable seal between the microelectrode and the membrane. However, in these cases (Fig. 2*f*) the translucent part of the cytoplasm, which is clearly visible as shown in Fig. 3, is rapidly redistributed in the cell after insertion of the electrode into the vacuole, and the potential rise coincides with the moment when the redistributing cytoplasm reaches the tip of the electrode (arrows in Fig. 2*f*). This observation perhaps suggests that the more negative potential obtained in Fig. 2*f* corresponds to E_{co} independent of the tonoplast potential whereas the less negative p.d. values observed initially with the electrodes apparently in the vacuoles of the ultracentrifuged cells are affected by the tonoplast potential. However, this still leaves three possible explanations of the E_{vo}^{app} thus obtained (*see above*, p. 310):

(*i*) it could correspond to the p.d. of the tonoplast alone, i.e. to the p.d. vacuole-medium unaffected by the plasmalemma;

(*ii*) it could correspond to the p.d. of a sort of tight junction between plasmalemma and tonoplast; and

(*iii*) it could correspond to E_{vo}^{real}, i.e. to tonoplast and plasmalemma in series.

Notwithstanding these ambiguities, we shall briefly consider the calculated E_{vc} values resulting from Eq. (1) under the alternative assumptions that $E_{vo}^{app} = E_{vo}^{true}$ in mildly and strongly centrifuged cells, respectively. In the following, p.d. measurements are taken from Table 2 to the nearest 5 mV: Eq. (1) $E_{vo} - E_{co} = E_{vc}$; mild centrifugation (1.6×10^3 *g*): 2 to 6 weeks $(-130) - (-130) = 0$ mV; ultracentrifugation (20×10^3 *g*): 2 to 6 weeks $(-65) - (-130) = +65$ mV; 7 to 9 weeks $(-60) - (-125) = +60$ mV.

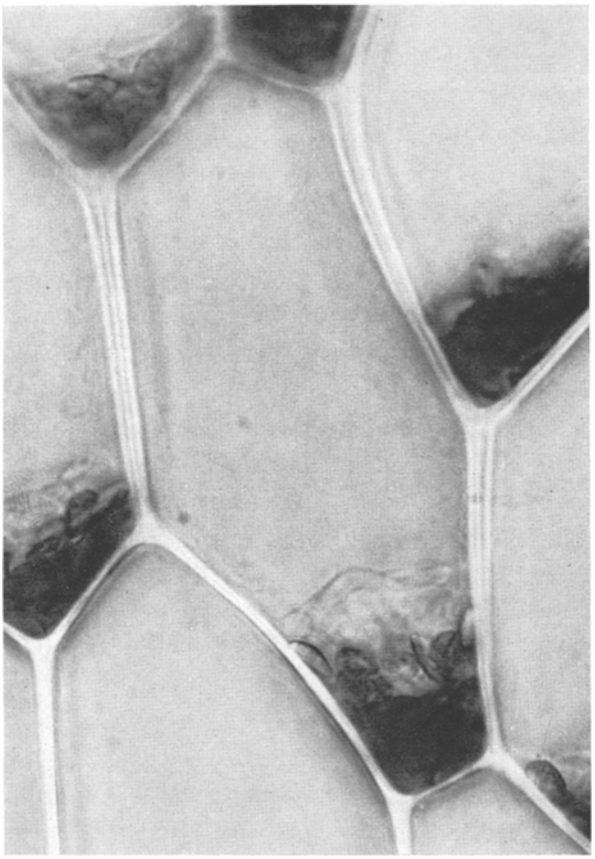

Fig. 3. Ultracentrifuged ($20 \times 10^3\ g$) *Mnium* leaf cells showing transparent cytoplasm on top of the sedimented chloroplasts. Migration of this transparent cytoplasm to the tip of the electrode may cause recordings like that shown in Fig. 2*f*. Magnification 900×

These calculated E_{vc} values can be compared with values suggested in the literature. In giant algal coenocytes the cytoplasmic layer along the cell wall is often thick enough to allow reliable measurements of E_{co} without making use of centrifugation. On the basis of such measurements and assuming that with the electrode in the center of the cell E_{vo}^{true} is measured, E_{vc} values of close to zero or slightly positive are obtained (i.e., 0 to +26 mV); E_{vo} in these cells is highly negative (summarizing tabulations: MacRobbie, 1970; Lüttge, 1973, p. 27). Similarly, it has been suggested that E_{vc} of higher plant cells is approximately zero (Etherton & Higinbotham, 1960; Denny & Weeks, 1968). This would be in agreement with our measurements, if after mild centrifugation $E_{vo}^{app} = E_{vo}^{real}$. Furthermore,

under all conditions $E_{cell} = E_{co}$ (Fig. 2). If E_{cell} measures E_{vo}, this would also show that $E_{vc} \simeq 0$. Of course, one would then have to think of an explanation for the lower E_{vo}^{app} observed after ultracentrifugation, perhaps suggesting more serious structural disarrangements, as discussed above (p. 311).

On the other hand, as pointed out in the introduction, it is also possible that E_{cell} measures E_{co}. Only in exceptional cases of algal coenocytes like *Valonia ventricosa* and *Chaetomorpha darwinii* E_{vc} is highly positive, i.e. +80 to +88 mV (*cf.* tabulations by MacRobbie, 1970, and Lüttge, 1973). This would be in agreement with our measurements if after ultracentrifugation $E_{vo}^{app} = E_{vo}^{real}$, which is suggested by tracings such as those of Fig. 2*f*. Since the assumptions that after mild centrifugation or after ultracentrifugation $E_{vo}^{app} = E_{vo}^{real}$ are mutually exclusive, this would then in addition require the assumption that the electrode apparently in the vacuole of the mildly centrifuged cells in fact measures E_{co} (*see above* p. 310). It also should be noted here that by contrast to our measurements, in *V. ventricosa* and *Ch. darwinii* E_{vo} is slightly positive (+10 to +17 mV).

Thus, in the *Mnium* cells equilibrated with APW, E_{vc} is either close to zero or +60 to +65 mV depending on the interpretation of the measurements. Although some of the other alternatives considered above have not been ruled out, they appear unlikely and there is no reason to suggest that under the conditions of our experiments highly negative potentials occur at the tonoplast. Therefore, it is quite probable that the high negative potential of the *Mnium* cells as was suggested for the algal cells is due to the plasmalemma (E_{co}).

Conclusions

As discussed above, the techniques described unfortunately do not allow more reliable measurements of E_{vo}^{true} of higher plant cells than provided in the literature so far. Thus, unambiguous calculations of E_{vc} cannot be made. On the other hand, the centrifugation techniques allow undebatable measurements of E_{co} of small cells of higher plants. We feel that this is an important improvement in studying ionic relations of higher plant cells. The techniques described here should also prove useful in investigations of the dependence of ion distributions on metabolic reactions using intercellular O_2- and pH-microelectrodes (*see* Bowling, 1973*a*, *b*).

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