A versatile means of intracellular labeling: injection of biocytin and its detection with avidin conjugates

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Biocytin is a biotin–lysine complex of low molecular weight containing about 65% biotin, which retains a high affinity for avidin. Since the latter molecule has been conjugated to several histochemical markers, the use of biocytin as an intracellular marker was investigated. Electrodes were filled with a solution of 4–6% biocytin dissolved in 0.5 M KCl and 0.05 M Tris buffer, pH 7.7. Neurons were recorded intracellularly in the supraoptic nucleus of an explant preparation of the rat supraoptico-neurohypophysial system and injected for 1–20 min with either hyperpolarizing or depolarizing current. Following variable recovery times, the explants were fixed in either 10% formalin or 4% paraformaldehyde overnight, sectioned on a vibratome, and incubated with the avidin–biotin complex (ABC) or avidin which had been conjugated to fluorescein, rhodamine, Texas Red or horseradish peroxidase and containing 1% Triton-X 100. A high percentage of injected neurons were recovered using each of the labels with about equal success. Both negative or positive current injection could be used with little electrode clogging. Labeling with fluorescent conjugates was qualitatively similar to that of Lucifer Yellow, whereas labeling with avidin coupled to horseradish peroxidase or with ABC was qualitatively similar to filling neurons directly with horseradish peroxidase. The advantages of this technique are the ease of injection of biocytin and the versatility in allowing the investigator to choose among light-emitting and light-absorbing images.

Introduction

Correlations between the physiological and morphological attributes of any single neuron are most directly studied by the intracellular injection of a marker following electrophysiological recording. This allows not only a precise determination of the recording site, but the study of the recorded neuron’s somatic, dendritic and axonal morphologies as well. Several substances have been used with varying success, and excellent reviews are available detailing their strengths and weaknesses (Nicholson and Kater, 1973; Kitai and Wilson, 1982; Stewart, 1978; Tweedle, 1978; Brown and Fyffe, 1984). Two currently popular and extremely useful markers for studies of mammalian neurons are Lucifer Yellow CH (LY) and horseradish peroxidase (HRP), and each has provided enormous growth in our understanding of the structure of physiologically identified neurons. Even though both markers meet the general requirements demanded of intracellular markers, including solubility in water, stability during histological processing, rapid diffusion and retention within the cell and high visibility either by itself or after a specific chemical reaction (Nicholson and Kater, 1973; Kitai and Wilson, 1982; Stewart, 1978; Tweedle, 1978; Brown and Fyffe, 1984). Two currently popular and extremely useful markers for studies of mammalian neurons are Lucifer Yellow CH (LY) and horseradish peroxidase (HRP), and each has provided enormous growth in our understanding of the structure of physiologically identified neurons. Even though both markers meet the general requirements demanded of intracellular markers, including solubility in water, stability during histological processing, rapid diffusion and retention within the cell and high visibility either by itself or after a specific chemical reaction (Nicholson and Kater, 1973; Kitai and Wilson, 1982; Stewart, 1978; Tweedle, 1978; Brown and Fyffe, 1984).
1973), they differ dramatically in their application and in the nature of the image provided.

LY renders a transient, fluorescent image with appropriate excitation and without further histochemical processing, is small enough (MW = 457) to pass gap junctions and is widely used to study the phenomenon of dye-coupling (Stewart, 1978). HRP produces a permanent, opaque image after histochemical reaction with a chromagen substrate and is suitable for correlated light and electron microscopic work. However, HRP is too large to pass gap junctions (MW about 40,000).

It is difficult to dispute the utility of either marker. Nonetheless, improvements in intracellular marking continue to be made. Carboxyfluorescein is more fluorescent than LY and can be used for dye coupling studies (Rao et al., 1986). The plant lectin phaseolus vulgaris leucoagglutinin (Sugiura et al., 1986) may be more useful than HRP for studying more distal axonal projections. The present paper details another labeling technique investigators may choose, the intracellular injection of biocytin, which is a complex formed of biotin and lysine. The salient features of biocytin are its ease of injection from unbevelled electrodes, and its high affinity for the egg white protein, avidin (Wright et al., 1952), which can be conjugated to a variety of useful markers. The latter feature allows versatility in producing either a light-emitting or light-absorbing image, and a resulting light microscopic image with a quality similar to those achieved with LY and HRP. A preliminary report of this technique has previously appeared in abstract form (Horikawa and Armstrong, 1987).

Materials and Methods

Preparation of solutions

The structure of biocytin (C_{16}H_{38}N_{4}O_{4}S) is shown in Fig. 1. The molecule is highly soluble in water and consists of about 65% biotin, the latter of which imparts a high affinity to the molecule for the egg white protein, avidin (Wright et al., 1952). Chemical analysis has revealed acidity constants for the carboxylic acid and amino groups of 2.26 and 9.29, respectively, an isoelectric point somewhere between 4.5 and 6.5, and behavior in some respects typical of a simple amino acid (Griesser et al., 1970).

Typically, biocytin (Sigma) was dissolved at 4–6% in 50 μl of 0.05 M Tris buffer, 0.5 M KCl, pH 7.0–7.6, although other solutions were also tried (see below). Solutions stored refrigerated could be useful for 2–3 days but later may have visibly precipitated. Fresh preparation is recommended with each experiment.

While it should be possible to influence biocytin's net charge and thus eject it selectively by varying the pH of the electrode solution, this has not been exhaustively tested. We had difficulty obtaining good recording when pH was dropped below 4.0 (when charge should be largely positive), as electrode resistance quickly rose to an unacceptable level. Alkaline solutions (when charge should be largely negative) with a pH as high as 9–10 were useful with hyperpolarizing current, but we have not exhaustively tested ejection at this pH using depolarizing current.

Electrophysiology

Electrodes were pulled from 1 mm glass containing a microfilament (A-M Systems) on a Narishige PE-2 vertical puller and backfilled using a microsyringe. The DC resistance of the electrodes used ranged from 30 to 200 MΩ. As viewed with a 100 x metallurgical objective, tip diameters ranged from 0.5 μm to something less than 0.25 μm (the limit of resolution). Penetration of neurons was achieved by passing brief current pulses through the electrode tip using circuitry provided with the intracellular recording amplifier (Neurodata). The polarity of these pulses could be changed and for a given impalement was the same as that used to inject the neuron.
Fig. 2. Examples of injection protocols. A: negative current injection. Five consecutive hyperpolarizing pulses (−0.35 nA, 220 ms, 1 Hz) and one depolarizing pulse (+0.35 nA) are shown. The depolarizing pulse was used to examine spikes if no anodal spike was present, and if the cell was not antidromically identified. In addition to the pulses, the cell was under a continuous hyperpolarization of −0.35 nA. B: positive current injection in a different neuron. In this case, the neuron was stimulated antidromically with a neurohypophysial shock (arrowhead), then depolarized with a 220 ms positive pulse (1.5 nA), at 1 Hz. A bridge imbalance is indicated by the differing heights of the antidromic and current-evoked spikes. Trace calibration: 50 mV, 50 ms.

Ejection of biocytin was attempted by either positive or negative current through the active bridge of the intracellular recording amplifier. Negative current consisted of 0.5–1 nA of continuous hyperpolarization with an additional 0.5 nA, hyperpolarizing, rectangular pulse (220 ms) at 1 Hz. The quality of the impalement was monitored by viewing membrane potential and anode break spikes or, if the latter were infrequent, by an occasional depolarizing pulse to view evoked spikes (Fig. 2). Positive current injections were made with a rectangular 220 ms depolarizing pulse of 0.5–2 nA at 1 Hz. Since at higher positive currents action potentials were often inactivated, impalement quality was primarily monitored by viewing the membrane potential and, when present, antidromic action potentials (Fig. 2B). Injections in stable recordings were terminated at 10–20 min. Otherwise, injection was stopped at the sign of deterioration in the recording. No hyperpolarizing current was used to stabilize the recordings when positive current was to be used for filling. In many neurons the input resistance of the membrane at rest was estimated from an I/V curve generated by passing 220 ms hyperpolarizing pulses (1 Hz) through the electrode.

The resistance and linearity of pipettes filled with biocytin, LY or only their respective vehicles, were compared. Thus electrodes were filled with 0.5 M LiCl, 0.5 M LiCl with 4% LY, 0.5 M KCl in 0.05 M Tris, and 4% biocytin in the latter solution (5 electrodes/group). Lissajous images generated through the x–y–oscilloscope from the amplifier output and a 10 Hz, adjustable triangular waveform were used to determine the linearity of the current passing ability of the electrode by visible deviation from the straight diagonal, in the absence of bridge balance (Park et al., 1983). Electrode resistance was then estimated by balancing the bridge. For these comparisons all of the electrodes were pulled at the same setting of the microelectrode puller, and only 15 min separated the pulling of the first and the last of the twenty electrodes used.

In vitro tissue preparation

Recordings were made from supraoptic nucleus (SON) neurons in an acutely maintained explant of the hypothalamo-neurohypophysial system, the preparation of which has been previously described (e.g. Armstrong et al., 1985; Armstrong and Sladek, 1985). Briefly, young adult male, Sprague–Dawley rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The brain was then excised and the ventral hypothalamus with the pituitary stalk and neurohypophysis were dissected free. The explant was then placed in a plexiglas recording chamber where it was perfused with warmed (32–33°C), oxygenated medium containing (in mM): NaHCO₃ 25, KCl 3, KH₂PO₄ 1.24, NaCl 124, glucose 10, CaCl₂ 2, and MgSO₄ 1.3. In about half the preparations, 25 ml of cold slice medium were perfused intracardially with the descending aorta clamped prior to dissection of the brain. This cleared the tissue of most erythrocytes, which can obscure parts of the labelled cell due to their reactivity with the histochemical reagents used to reveal HRP.

A bipolar stimulating electrode made of 0.005 inch insulated nichrome wire was placed on the pituitary stalk and used to elicit antidromic action potentials with monophasic stimuli of 0.5 ms,
4-20 V. Recordings were not begun until at least 2 h had passed from the time of dissection.

**Histology**

The tissue was fixed overnight by submersion in cold (4°C) 10% formalin or 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.3, 15-120 min following the injection. Explants were sectioned on a vibratome at a thickness of 60-75 µm. Sections were collected in PBS and rinsed several times before continuing.

Injected cells were identified by incubating the tissue 1-4 h in avidin which had been conjugated to various markers, including horseradish peroxidase (A-HRP), Texas Red (A-TR), fluorescein (A-F1), and rhodamine600 (A-Rh), or in the avidin–biotin–HRP complex (ABC solution) in dilutions ranging from 1:100-1:400 in PBS. All of these reagents were obtained from Vector Labs. Initially, the tissue was pretreated for 1-2 h in a 0.5-1% solution of Triton-X100 in PBS. More recently the markers have been diluted in the detergent solution directly without any pretreatment. As far as we can determine it is necessary to use the Triton-X (or some other means of permeabilizing cell membranes) to achieve full labelling.

After rinsing in several changes of PBS over 1 h, the sections were prepared for microscopic examination. Sections containing fluorescent markers were most often mounted in a solution of glycerol : PBS (1 : 3) containing 1% of the antioxidant n-propylgallate to inhibit photobleaching (Rao et al., 1986). Sections were sometimes cleared in 100% solutions of glycerol or dimethylsulfoxide. A Nikon epifluorescence illumination system was used to examine the tissue. The G-2A filter was used to examine A-Rh and A-TR and the B-2A filter set was used for A-F1. Fluorescent images were photographed using Kodak Tri-X pan film (ASA 400).

Sections incubated with A-HRP or ABC were rinsed and reacted with diaminobenzidine (DAB) (0.06%) and H$_2$O$_2$ (0.003%) in 0.15 M tris buffer for 10 min. The sections were thoroughly rinsed and either mounted onto gelatin-coated slides or placed in 0.5% osmium tetroxide for 15 min. In the former case sections were air dried, defatted and overslipped with Permount. Osmicated sections were rinsed in deionized water, dehydrated in alcohols, mounted from xylene onto a drop of Permount placed on a slide and coverslipped. Osmication minimized tissue shrinkage and additionally intensified the reaction product due to the osmiophilic nature of the DAB reaction product.

**Results**

Injections were made in 39 cells from 20 explants and were associated with recordings ranging, by our criteria, from poor (membrane potentials < 40 mV, spikes < 50 mV and broad) to excellent (membrane potentials > 40 mV, spike heights > 50 mV and narrow). Neurons were recovered in both the principal or anterior (SONa) and tuberal (SONt) portions of the SON within the explant. The membrane input resistance measured in the better recordings ranged from 100 to 250 MΩ.

**Injection parameters**

Except when using acidic buffers, we were able to eject biocytin with either depolarizing or hyperpolarizing current with virtually no electrode clogging. Most attempts were made with a pH of 7-7.6, because we found the electrode quality uniformly high and clogging minimal. Of these, 5 of 8 attempts with depolarizing current resulted in filling, whereas 11 of 14 attempts with hyperpolarizing current were successful. There was no noticeable difference in the quality of filling between the two injection modes.

Severe problems with electrode resistance and clogging were encountered when acidic solutions were used, and most electrodes became quickly unusable when initially passing through the tissue and attempting to impale cells. Both cells injected with positive current in low pH (2.5–3.5) were labelled, whereas only one unsuccessful attempt was made with hyperpolarizing current. Filling solutions of more alkaline pH were not extensively tested with depolarizing current (1 of 3 cells injected with depolarizing current was recovered),
but 9 of 12 neurons were successfully injected with hyperpolarizing current. Although not quantified, the quality of filling depended upon the length of injection, impalement quality and the time passing between time of injection and time of fixation. The best fills were

Fig. 3. Photomicrographs of supraoptic neurons which had been injected with biocytin and revealed with avidin conjugated to various fluorescent markers. All sections were cut in the horizontal plane. A: a large multipolar SONt neuron within a 75 μm section stained with A-FI. The neuron had been antidromically identified as projecting into the neurohypophysis. Three fine processes (arrows) emerge from the soma, as do 4 other larger processes, one of which is indicated with a curved arrow since its origin is out of focus. The fine processes could not be traced far from the neuron. The small arrow on the right indicates a fluorescent artifact of undetermined nature, but which was not associated with the neuron. B: unidentified multipolar neuron near the SONt within a 60 μm section, stained with A-Rh. The nucleus (asterisk) has sequestered the stain. The arrow points to a varicosity at the end of a short thin process which appears to end within the section. C: an unidentified bipolar neuron stained in the region dorsal to the SONa in a 60 μm section. The two stained processes both looked like dendrites, having a moderate number of spines. D: lower power view of same cell in C, showing its relationship to the optic chiasm (oc). E: an antidromically identified SONt neuron revealed with A-FI. The axon (larger arrows) could be traced medially into the neurohypophysial tract after first projecting rostrally. The dotted line indicates the edge of the tissue section. Scale: 50 μm in A, B and C; 100 μm in D and E. In A-E, rostral is top, caudal is bottom, lateral is left and medial is right. Inset: the traces illustrate the collision test for the cell in E. In the top trace, a 10 ms current pulse which failed to elicit a spike was followed by a shock (vertical arrowhead) to the neurohypophysial stalk, which elicited an antidromic spike. When the current pulse was slightly elevated to reach threshold for a spike, as shown in the middle trace, the antidromic spike was blocked (indicated by asterisk). In the lower trace, a spike initiated by a 20 ms pulse preceding that shown in the middle trace failed to block the antidromic spike, indicating it occurred outside the collision interval for the neuron. Trace calibration: 20 mV, 20 ms.
obtained from neurons injected 5–20 min which
demonstrated the more negative membrane poten-
tials and larger action potential heights. Poorer
recordings were associated with weak labelling
unless the tissue was fixed within 30 min of injec-
tion, whereas good recordings were associated with
good filling even when the tissue was fixed hours
after injection. This suggests a leakage of the dye
from poorly impaled or unhealthy neurons.

As far as could be determined there was little ill

Fig. 4. Photomicrograph of a 60 μm horizontal section through the anterior, principal part of the SON, showing a neuron which had
been filled with biocytin. The neuron was revealed by incubation of the tissue with A-HRP, followed by the histochemical
localization of HRP, then treatment with osmium tetroxide. A and B represent medium (A) and high (B) magnification views. In A,
shorter arrows are pointing to dendrites, and the longer arrow to the axon as it emerges from the soma. The right dendrite leaves the
section and was found intact in an adjacent section. Processes from another neuron filled nearby, from a separate injection, are
shown with arrowheads. In B, the crisp outline of the soma and processes is more readily appreciated. Again, arrowheads indicate
processes from another neuron. In both photos, anterior is at the top, medial to the right, lateral to the left, and posterior to the
bottom. oc, optic chiasm.
effect of injecting biocytin, although neurons were not typically recorded for more than 2 h. Certainly, there was no short term, dramatic effect on membrane potential or in the ability to generate action potentials. For some SONt neurons the neuron was tested to α1-adrenoceptor agonists (Armstrong et al., 1986), and the response, a consistent excitation, was similar to uninjected cells.

Labelling alternatives

Injected neurons were recovered with each of the avidin-based reagents with about equal facility. Injected neurons reacted with the fluorescent conjugates are shown in Fig. 3. The images produced by A-TR and A-Rh exhibited much less photobleaching than those of A-Fl, even without the use of n-propylgallate. The addition of this antioxidant considerably reduced the photobleaching of all the fluorescent labels. The use of A-HRP or ABC resulted in an image not unlike those published for the intracellular injection of HRP (Fig. 4). Small diameter processes and spines were observable, indicating adequate diffusion of the biocytin throughout the cell. Axons of well-filled SONt neurons could be traced into the hypothalamo-neurohypophysial tract more than a 1 mm away. The best dilution of label was usually 1 : 200.

Electrode properties

While there were no significant differences in the DC resistance or linearity between LY- and biocytin-filled electrodes (Table I), some other differences were noted. Electrodes filled with the 0.5 M KCl and Tris, but without biocytin, were significantly less linear than those of the other 3 groups, and had less resistance than electrodes filled with only LiCl. Recordings with biocytin-filled electrodes were not particularly noisy and were useful for observing both slow (e.g. postsynaptic potentials, drug-induced depolarizations) and fast events such as action potentials (Fig. 3E, inset).

Dye-coupling

Although biocytin is certainly small enough to pass through gap injections, we were unable to determine whether it would be useful to study dye-coupling. In only a few cases was more than one neuron labelled after a single injection. However, in one particular explant we used capacitance feedback to try to impale cells, and noted many neurons were labelled which did not necessarily have somata or dendrites in close proximity to one another. Thus this method of penetrating cells should be used with caution.

Discussion

The chief advantages of using biocytin for intracellular marking are its small size, high solubility in water, and affinity for avidin. The first two allow for its ease of injection during intracellular recording from the small tips of micropipettes. Biocytin's high affinity for avidin imparts a sensitive means of detection using either light-emitting or light-absorbing markers. In addition, labelling with biocytin satisfies other general requirements demanded of intracellular markers, such as rapid

| Resistance and linearity of electrodes filled with LY, biocytin or their vehicles |
|---------------------------------|------------------|------------------|------------------|
| 0.5 M LiCl + 4% LY + 0.5 M LiCl | 0.5 M KCl + 0.05 M Tris | 4% biocytin + 0.5 M KCl + 0.05 M Tris |
| Resistance (MΩ) | 70.5 + 8.1 | 67.8 + 1.7 | 52.8 + 3.7 ** |
| Linear to (nA) | 2.1 + 0.1 | 2.1 + 0.3 | 1.3 + 0.1 ** |

ANOVA for resistance: $F = 3.37, P < 0.05\%$; ANOVA for linearity: $F = 8.12, P < 0.005\%$.

* Newman–Keuls: $P < 0.05\%$, smaller than 0.5 M LiCl only.

** Newman–Keuls: $P < 0.01\%$, smaller than all other groups.
diffusion within the cell, low toxicity, minimal extracellular leakage and stability with histological processing (Nicholson and Kater, 1973). Since labelling using biocytin would share some features of using LY and HRP, it is worth making a more detailed comparison with these two markers.

LY has essentially replaced the previously popular fluorescent marker Procion Yellow (Stretton and Kravitz, 1968), because it is more brilliant, diffuses more rapidly and is more easily ejected from pipettes (Stewart, 1978). LY-filled neurons can be viewed in living and fixed tissue without further histochemical treatment, an advantage over both HRP and biocytin. It appears to cross gap junctions and is widely used to study the phenomenon of dye-coupling among neurons, which can be indicative of electronic coupling. Staining with LY is compatible with immunofluorescent (e.g. Reaves and Hayward, 1979) or immunoperoxidase (Smithson et al., 1984) labelling of neuronal antigens in the same tissue section. LY is easily passed with small, hyperpolarizing currents from small electrode tips, a feature it should share with biocytin due to their small size. The chief disadvantages of LY are its transience due to fading, electron lucence (although see Maranto, 1982, for LY's adaption to electron microscopy), and, in practice, a high electrode resistance (100–500 MΩ) limiting current passing ability. The latter feature is avoidable and probably related to the fact that investigators reporting such high resistances dissolved LY in water only or in salt concentrations even lower than 0.5 M (e.g. Andrew et al., 1981; Grace and Llinas, 1985; Katz, 1987; Randle et al., 1986). In the present study LY was found to contribute little to the resistance of the LiCl solution in which it was dissolved. However, in our experience LY electrodes can clog much more easily than biocytin-filled electrodes during injection.

With regards to the transience of the fluorescent markers used with biocytin, fading can be attenuated by the use of A-Rh or A-TR and by adding antioxidant to the mounting media. The compatibility of biocytin labelling with immunohistochemistry has yet to be studied, but there is no reason a priori to suspect that double labelling could not be achieved using two distinguishable fluorochromes. However, our preliminary attempts suggest that the immunocytochemistry should be performed before localizing the biocytin.

Although dye-coupling of SON neurons following LY injections in slices has been reported (Andrew et al., 1981; Cobbett and Hatton, 1984), previous investigators found no coupling using an explant preparation similar to the one described here (Randle et al., 1986; Reaves et al., 1983). While there is no explanation for this discrepancy, the transfer of biocytin among coupled cells needs to be thoroughly examined in a different system. Thus, LY still has much to recommend it, and like cobalt (Pitman et al., 1971) or nickel-lysine (Fredman, 1987) would certainly be preferable for wholemount preparations typical of invertebrate studies and for examining living neurons in culture (Gähwiler, 1981) or in acutely prepared explants of various types (e.g. Miller and Selverston, 1979; Katz, 1987).

Bennet (1973) used HRP as an intracellular marker in his studies of junctional complexes, and may have been the first to inject it into invertebrate neurons. As for identifying recorded mammalian neurons, Lynch et al. (1974) made iontophoretic injections following extracellular recordings and recovered filled neurons after reacting fixed sections with benzidine. The application of this technique to intracellular injection was made a short time later by several investigators, in several preparations, working independently (Cullheim and Kellerth, 1976; Snow et al., 1976; Jankowska et al., 1976; Kitai et al., 1976; Light and Durkovic, 1976; Muller and McMahan, 1976).

The attributes of the intracellular HRP technique in its present form are reviewed by Kitai and Wilson (1982). Chief among them is the formation of a stable, highly resolvable light and electron opaque image of the injected neuron with a simple histochemical reaction. Several chromogens are available, but typically DAB is used, due largely to its stability in both water and organic solvents and the non-crystalline, osmophilic nature of the reaction product. The same neuron can be easily studied with the light and electron microscope with good ultrastructural preservation. HRP has been injected with success
using either pressure or with relatively low depolarizing currents (1–2 nA). However, its size is several orders of magnitude larger than LY and bevelled or broken tips are recommended to facilitate its passage from the electrode tip. The large size precludes passage of the HRP through gap junctions, an advantage in reducing doubt about the recorded neuron, but obviously preventing its use in the study of dye-coupling. A second feature of the HRP technique is that immunocytochemical labelling of the recorded neuron, although not often attempted, seems presently limited to electron microscopy (Somogyi and Soltesz, 1986) or possibly to the use of sections thin enough to observe the cell in more than one section, since the opaque reaction product obscures a second label in the same section under the light microscope. Finally, investigators have had difficulty recovering neurons after survival times long enough to allow diffusion to the full extent of the axonal projection, due either to the eventual toxicity of HRP or to damage from current injection (although see Tamamaki et al., 1984).

While biocytin's ease of injection from unbevelled microelectrode tips is one advantage over HRP, the necessity of permeabilizing membranes to achieve penetration of the large avidin conjugates undoubtedly means that ultrastructural preservation would suffer. Although we have not examined any stained cells with the electron microscope, we expect the morphology to suffer much the same as with detergent treatments for immunocytochemistry. Thus, while the light microscopic images achieved with A-HRP are comparable to those with the intracellular injection of HRP, the latter technique would probably be the one of choice for electron microscopic studies. Nevertheless, preservation may be adequate to study some ultrastructural aspects of the labelled neuron, and further attempts should be made to achieve penetration while minimizing damage to membranes, such as with freeze thawing (Kosaka et al., 1986).

In general, our studies on the electrode properties of biocytin-filled electrodes indicate that it did not significantly increase electrode resistance over vehicle alone, but improved the linear response of these KCl-Tris electrodes. The biocytin-filled electrodes were as a group no different than electrodes filled with LY, and were quite adequate for studying the membrane properties of SON neurons.

The ability to successfully eject biocytin with both hyperpolarizing and depolarizing current with the same buffer is somewhat of a disadvantage, since many neurophysiologists prefer to use hyperpolarizing current at some stage after impalement to help stabilize the recording, and this risks staining a neuron which may be impaled only briefly. The same limitation is also true with LY, but not HRP, which can be selectively ejected with positive current. The wide range in pH between the amino and carboxylic acidity constants suggests that a stronger buffer at either pH extreme will be necessary to influence biocytin's net charge enough for selective iontophoresis. An acidic vehicle which reduced the clogging problem may yet be useful for positive current injections and deserves further study.

In conclusion, while biocytin is not offered as a replacement for either HRP or LY, some investigators may find it an attractive alternative for labelling neurons for light microscopy. Its chief advantages are the ease of injection and the ability to choose between fluorescent and opaque images.

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