Published June 1, 1970

HISTOLOGICAL STAINING PROPERTIES OF PROCION YELLOW M4RS

B. W. PAYTON. From the Faculty of Medicine, Memorial University of Newfoundland, St. John's, Newfoundland, Canada

INTRODUCTION

Procion Yellow M4RS (Imperial Chemical Industries, America Inc., Stamford, Conn.) has recently been described as a useful fluorescent dye for the injection and marking of single neurons (2, 5, 7). Its advantage in this respect appears to be mainly due to its inability to diffuse out of such injected cells and its subsequent binding with cytoplasm. This staining of intracellular material survives normal histological fixation and embedding procedures. The inability of the dye to cross such membranes suggested that it could be used to mark extracellular space when applied as a vital stain to isolated tissues, and that it might also provide a good indication of the sites of epithelial diffusion barriers. Some of the in vivo and in vitro biological staining properties of Procion Yellow and other related Procion dyes have already been described (1) and, because of the irreversible nature of the staining process, these dyes have been of value as permanent markers in growth processes of teeth and bone.

This report describes experiments done with a number of tissues including frog sciatic nerve, a frog nerve-muscle preparation, crayfish nerve cord, frog retina, and toad bladder. The results show that Procion Yellow does fail to penetrate many cells and that it has certain additional advantages as a histological stain. Thus Procion revealed striking differences in the diffusion barriers offered by the sciatic nerve of the frog and the crayfish cord and between the membranes of myelinated nerve fibres, muscle fibres, intrafusal fibres, and epithelial cells of the toad bladder.

MATERIALS AND METHODS

To stain isolated tissues, the tissues were bathed in the appropriate Ringer's solution containing 1% Procion Yellow, diluted to maintain equiosmolarity with normal Ringer's, for 1–2 hr at room temperature (20°C). The dye solution was then washed out for 15–30 min with normal Ringer's or immediately replaced with fixative (2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, 430 milliosmols). The fixed tissues were dehydrated with alcohol, embedded in Epon 813 or Maraglas 732, and sections 0.5–2.0 μ thick were cut with a glass knife. Fluorescence microscopy was carried out with a Zeiss BG 12 activation filter (Carl Zeiss, Inc., New York, N. Y.) and orange barrier filters. Background autofluorescence of unstained material following such fixation is green to yellow in color.

Published June 1, 1970

RESULTS

Isolated frog sciatic nerve preparations treated in this way showed no staining of the nerve fibres when the perineurium was intact, though the connective tissue in the perineurium showed marked Procion fluorescence (orange-pink). This contrasted with the yellowish autofluorescence of the myelin (Fig. 1 *A*). In contrast to these results, when the nerve sheath was split the epineurium was stained and, following bleaching of the background autofluorescence by continued light activation, definite Procion staining of the myelin could also be observed (Fig. 1 *B*). No axoplasmic staining was detected but the cytoplasm and nuclei of Schwann cells were stained. During the staining of desheathed nerves, conduction block occurred; this block was reversible on return to normal Ringer's solution. Procion did not cause conduction block of sheathed nerves.

When frog nerve-muscle preparations (extensor digitorium longus IV) were treated in a similar manner, twitch responses to motor nerve stimulation failed within 15 min, and muscle spindle discharges, as monitored by afferent action potentials in the nerve following stretch, were also abolished. Both effects were reversible on returning to normal Ringer's solution. Sections of the muscle showed Procion staining of the connective tissue surrounding the nerve bundle and of that between the muscle fibres (Fig. 1 *C*). No staining of the myoplasm of the twitch fibres was visible except in damaged fibres which could be identified by the usual retraction clots (6). In contrast, sections of muscle spindles showed clear intracellular staining of the intrafusal fibres (Figs. 1 *C* and 1 *D*). In longitudinal sections this staining was seen to be more intense in the neural portion of the spindle.

Isolated nerve cords of crayfish, consisting of four or five ganglia and their connectives, could also be stained in this manner (3). A reversible local anesthetic action of the dye on this preparation was also observed. The sheath surrounding the nerve cord was ineffective as a barrier to penetration of the dye. Intense fluorescence staining of the outlines of all the axons within the connective was seen (Fig. 1 *E*). These axons are surrounded by loose layers of Schwann cell membrane. At the connective level very little axoplasmic staining was observed. Such staining was observed, however, in a number of axons sectioned at the ganglion level. This axoplasmic staining could be accounted for by diffusion of the dye along the axons from the cut peripheral branches which leave and enter the cord at the level of the ganglion and whose cut ends were lying free in the bathing media. The cytoplasmic staining of some of the nerve cell bodies at the ganglion level may also be explainable in this way. As expected, the giant fibres which have no such branches (5) were free of cytoplasmic staining.

On the other hand, neural staining of isolated tissues of frog central nervous system showed clear evidence of intracellular staining. For example, the retinae of freshly removed frog eyes were stained by removing the cornea, lens, and vitreous humor and immersing them in 1% Procion-frog Ringer's. No attempt was made to monitor any physiological activity of such preparations. Definite staining of all neural elements in these retinae, including ganglion cells, bipolar cells, rods, cones, and myelinated optic nerve fibres, was seen. It is possible that, owing to anoxia, the permeability of the membranes of these cells was altered and allowed intracellular staining.

In two experiments, isolated toad bladders were either filled or immersed in a Ringer-Procion solution; there was no connection between the luminal contents and the solution bathing the serosa. Staining of the epithelial cells was only seen when the dye was in direct contact with these cells. When the serosal surface was in contact with the dye solution, staining of the connective tissue was seen to extend as far as the basal lamina of the epithelial layer and no farther. These bladders continued to show rhythmic contractions throughout the staining procedure. The diffusional barrier to Procion in this tissue is at the epithelial cell layer, which is well known to be a barrier to many ions.

Red blood cells seen in sections of these preparations showed no intracellular staining. Similarly, human blood incubated with 0.1% Procion Yellow for 24 hr did not show cytoplasmic fluorescence, except for numerous pinocytotic vesicles in some of the white cells. The cytoplasm of these cells, however, could be stained following fixation of a smear with methyl alcohol.

In addition to its use in staining isolated tissues, Procion Yellow was found to stain sections of tissues embedded in Epon or Maraglas. Although a number of methods are available for staining tissues embedded in this way, many involve a number of steps (3). Like toluidene blue, Procion Yellow can be used as a simple aqueous solution,

Published June 1, 1970

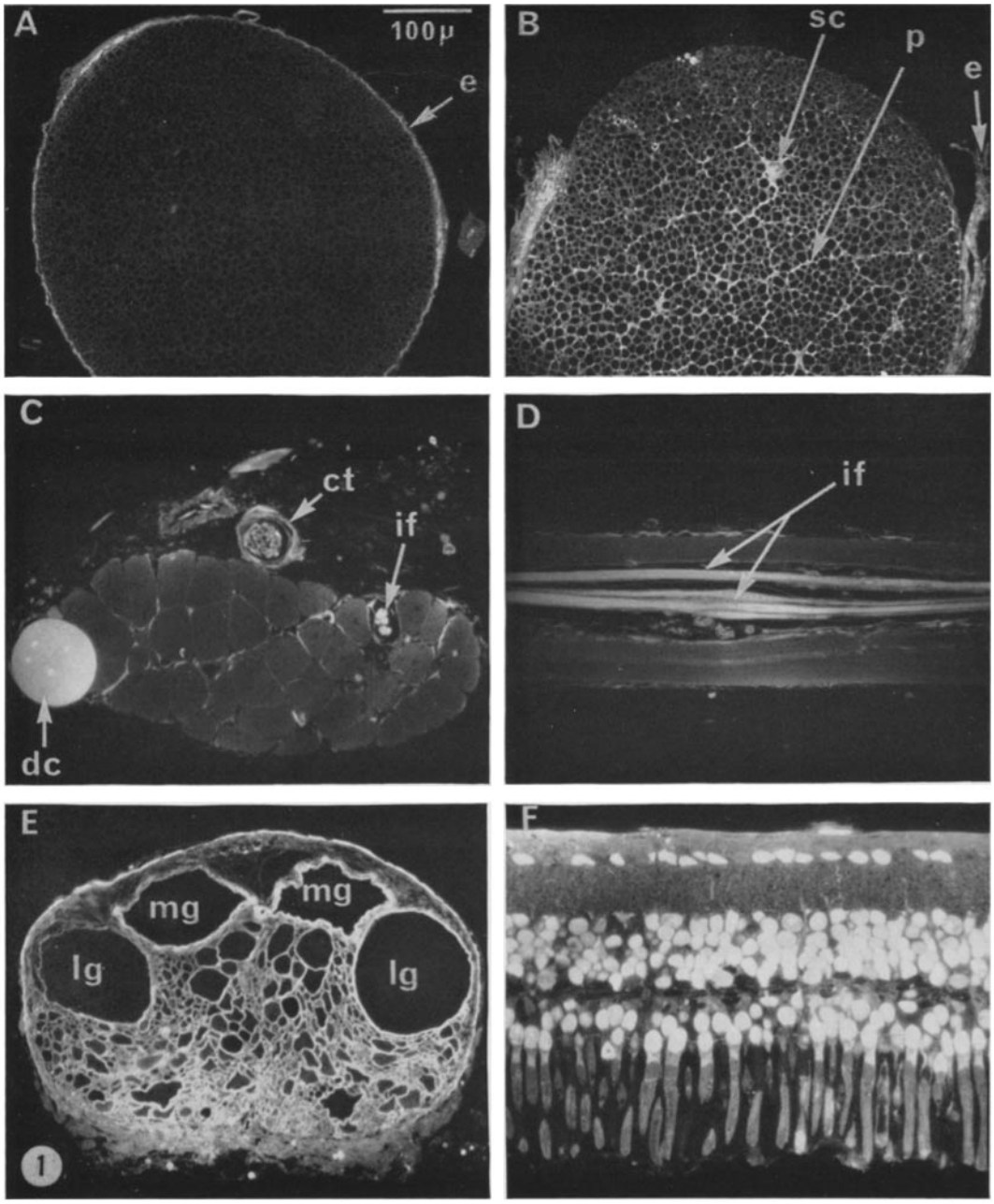

FIGURE 1 *A*. Transverse section through frog sciatic nerve. Only the epineurium (*e*) shows fluorescent staining by Procion Yellow. The axons show autofluorescence. *B*. After opening the epineurium (*e*), the perineurium (*p*) and myelin show fluorescent staining. Dye has entered the Schwann cell (*sc*) but not the axons (same photographic exposures for *A* and *B*). *C*. Transverse section of the extensor digitorum longus muscle of the frog, showing fluorescent staining in connective tissue surrounding the nerve axons (*ct*) and between muscle fibres. The damaged cell (*dc*) is stained, so are the intrafusal fibres in the muscle spindle (*if*). *D*. Longitudinal section through a muscle spindle showing the intracellular staining of the intrafusal fibres (*if*). *E*. Transverse section through the connective of a crayfish nerve cord. Some fluorescent staining of the cytoplasm of the smaller axons is seen, but none is seen in the lateral (*lg*) or medial (*mg*) giant fibres. *F*. A Procion-stained, Epon section of frog retina. As in *E*, the whole tissue shows fluorescent staining, and an intense staining of the nuclei of these cells is seen; the rod inner segments are more intensely fluorescent than the outer segments.

but staining times nceessary for best results are much longer, 1–2 hr, and staining is ineffective if the preparation has been previously postfixed in osmium tetroxide. An illustration of a 1 μ section of frog retina embedded and stained in this way is shown in Fig. 1 *F*. The resolution that can be obtained by staining 0.5–1 μ sections is enhanced by the fluorescent nature of the stain, though with thinner sections this is limited by the light intensity of the fluorescence.

These results and those obtained by intracellular injection methods (3) suggest that Procion Yellow may bind with certain cell membranes which remain impermeable to it. They also suggest that it can be a useful compound in the study of diffusional barriers such as the blood-brain barrier. In addition, as a conventional stain it can have some advantages when high resolution is required.

I thank Dr. P. G. Model and Dr. G. D. Pappas for helpful discussion. Part of this work was carried out in the Department of Anatomy, Albert Einstein College of Medicine, New York.

Received for publication 14 November 1969, and in revised form 19 December 1969.

REFERENCES

1. GOLAND, P. P., and N. G. GRAND. 1968. Chloro-s-triazines as markers and fixatives for the study of growth in teeth and bones. *Amer. J. Phys. Anthropol.* **29**:201.
2. KRAVITZ, E. A., A. O. W. STRETTON, J. ALVAREZ, and E. J. FURSHPAN. 1968. Determination of neuronal geometry using an intracellular dye injection technique. *Fed. Proc.* **27**:749.
3. PAYTON, B. W., M. V. L. BENNETT, and G. D. PAPPAS. 1969. Permeability and structure of junctional membranes at an electrotonic synapse. *Science (Washington).* **166**:1641.
4. PEASE, D. C. 1964. *In* Histological Techniques for Electron Microscopy. Academic Press Inc., New York. 256.
5. REMLER, M., A. SELVERSTON, and D. KENNEDY. 1968. Lateral giant fibers of crayfish: Location of somata by dye injection. *Science (Washington).* **162**:281.
6. SPIEDEL, C. C. 1939. Studies of living muscle. II. Histological changes in single fibers of striated muscle during contraction and clotting. *Amer. J. Anat.* **65**:471.
7. STRETTON, A. O. W., and E. A. KRAVITZ. 1968. Neuronal geometry: Determination with a technique of intracellular dye injection. *Science (Washington).* **162**:132.