by paper chromatographic procedures (Fig. 2).

Activity of brain ADH was similar in a number of respects to the liver activity. Their apparent $K_m$ values for ethanol, NAD+, and lactaldehyde were comparable, and both exhibited clear and quantitatively similar stereospecificity in regard to the D- and L-isomers of lactaldehyde (Table 1). Two different types of inhibitor of ADH, pyrazole and sulfide (*9*), inhibited the velocities of the brain and liver reactions to almost identical degrees (Table 2). Native activity in liver, however, exceeded that of brain by several orders of magnitude; the maximal rate of ethanol oxidation in liver was 9 mmole/g of liver per hour in contrast with 2.4 μmole/g per hour in brain.

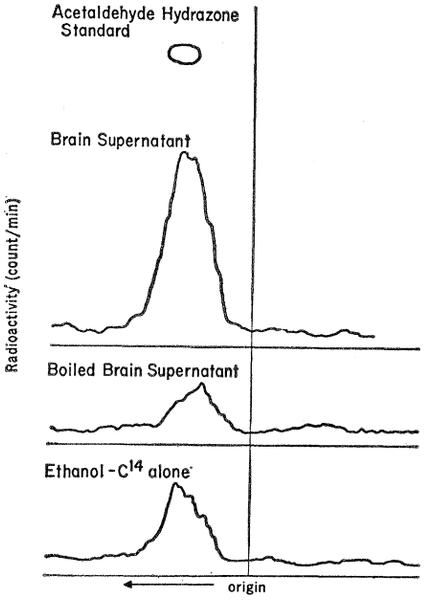


Fig. 2. Identification of acetaldehyde as the product of the reaction used for the assay of ADH in brain. Reaction mixtures were the same as those described in Fig. 1, except that the ethanol content was 200 m*M* ethanol-1-$^{14}$C (specific activity, 0.5 mc/mmole). The enzyme content was 0.2 ml of the 100,000*g* supernatant portion of brain. Incubation time at 37°C was 2 hours. Reactions were carried out in sealed Thunberg tubes, the sidearms of which contained 0.5 ml of 0.01*M* 2,4-dinitrophenylhydrazine hydrochloride. At the end of the incubation contents of the sidearms were extracted into chloroform, and the extracts were then evaporated to dryness to remove unreacted ethanol-$^{14}$C. The residue was redissolved in 0.3 ml of chloroform, and 5-μl samples were chromatographed on Whatman No. 1 filter paper impregnated with phenoxyethanol in parallel with an authentic standard of the 2,4-dinitrophenylhydrazone of acetaldehyde. The chromatogram was developed in phenoxyethanol-saturated heptane by the descending chromatographic method of Lynn *et al.* (*7*). Strip-scanning revealed a single radioactive peak that migrated the same distance from the origin as the yellow spot of an authentic preparation of the 2,4-dinitrophenylhydrazone of acetaldehyde (top two panels) (*8*). A similar but much smaller peak, 22 percent of the first, was detected in boiled enzyme controls, but was probably derived from acetaldehyde-$^{14}$C contamination of the ethanol-$^{14}$C, since a peak of comparable magnitude was obtained by incubating ethanol-$^{14}$C alone in water (lower two panels).

The relatively low level of activity of brain ADH raises the question of its physiological significance. It should be noted that metabolic capacities of tissues estimated on the basis of in vitro enzyme assays need not reflect their actual activities in vivo. For example, in the intact adult rat ethanol is metabolized at a rate of 1.0 to 1.5 μmole/hour (*10*) despite a hepatic capacity to oxidize 9 mmole of ethanol per hour per gram of tissue. The liver alone, however, does account for at least 90 percent of the total body metabolism of ethanol (*11*), and the brain probably plays a minor role in the body's disposition of an ethanol load. A cerebral mechanism for the oxidation of ethanol may be important for local adjustments during exposure to ethanol; or, since alcohol dehydrogenases of low substrate specificity are found in abundance in the tissues of many organisms that never come into contact with ethanol (*12*), Theorell has suggested that ADH functions in the metabolism of some as yet unidentified physiological substrate (*9*). Sustained tissue burdens of ethanol may affect the steady-state levels of this physiological substrate; such alterations may occur in brain and may be relevant to understanding the neural disorders associated with prolonged alcohol ingestion or withdrawal.

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**References and Notes**

1. C. T. Beer and J. H. Quastel, *Can. J. Biochem.* **36**, 543 (1958); J. C. Towne, *Nature* **201**, 709 (1964); N. C. R. Räihä and M. S. Koskinen, *Life Sci.* **3**, 1091 (1964).
2. N. K. Gupta and W. G. Robinson, *Biochim. Biophys. Acta* **118**, 431 (1966).
3. E. Huff, *ibid.* **48**, 506 (1961).
4. A. Nyberg, J. Schuberth, L. Änggard, *Acta Chem. Scand.* **7**, 1170 (1953).
5. E. Huff and H. Rudney, *J. Biol. Chem.* **234**, 1060 (1959); B. Zagalak, P. A. Frey, G. L. Karabatsos, R. H. Abeles, *ibid.* **241**, 3028 (1966).
6. L. R. Jones and J. A. Riddick, *Anal. Chem.* **29**, 1214 (1957).
7. W. S. Lynn, L. A. Steele, E. Staple, *ibid.* **28**, 132 (1956).
8. The melting point of the prepared 2,4-dinitrophenylhydrazone of acetaldehyde was 167°C; the theoretical melting point is 168.5°C.
9. H. Theorell, *Harvey Lec. Ser.* **61**, (1965–66), 17 (1967); J. von Wartburg, J. L. Bethune, B. Vallee, *Biochemistry* **3**, 1775 (1964).
10. J. C. Aull, Jr., W. J. Roberts, Jr., F. W. Kinard, *Amer. J. Physiol.* **186**, 380 (1956); F. W. Kinard, G. H. Nelson, M. G. Hay, *Proc. Soc. Exp. Biol. Med.* **92**, 772 (1956); A. Mikata, A. A. Dimakulangan, W. S. Hartroft, *Gastroenterology* **44**, 159 (1963).
11. E. Jacobsen, *Pharm. Rev.* **4**, 107 (1952).
12. H. Sund and H. Theorell, in *The Enzymes*, P. D. Boyer, H. Lardy, K. Myrbäck, Eds. (Academic Press, New York, 1963), vol. 7, p. 25.

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## Neuronal Geometry: Determination with a Technique of Intracellular Dye Injection

Abstract. *In a study of the specificity of neuronal connections in lobster abdominal ganglia, the dye Procion Yellow M4RS was electrophoretically injected into identified cell bodies. This dye spreads into fine branches of cells, survives fixation and routine histological procedures, and permits the reconstruction of cell shapes through examination of serial sections of ganglia. Certain cells were found to have an internal bilateral symmetry. Repeated injection of the same cells in ganglia from different animals showed that cells have characteristic shapes and that the neuropil is highly structured. This method of dye injection should have general applicability in studies where a knowledge of the geometry of specific cells is important.*

In a consideration of how a structure as complicated as a nervous system is built, one of the most basic questions is how accurately are the elements of the system, the neurons, connected together. Is there a well-defined circuit diagram for each nervous system? Are the same cells always connected in the same way?

A great aid in answering such questions may arise from recent studies in a variety of invertebrates and in a primitive chordate (*1, 2*) where it has been possible to identify and tabulate individual cells, find the same cells in different individuals of the same species, and establish maps of the positions of cell bodies of known function.

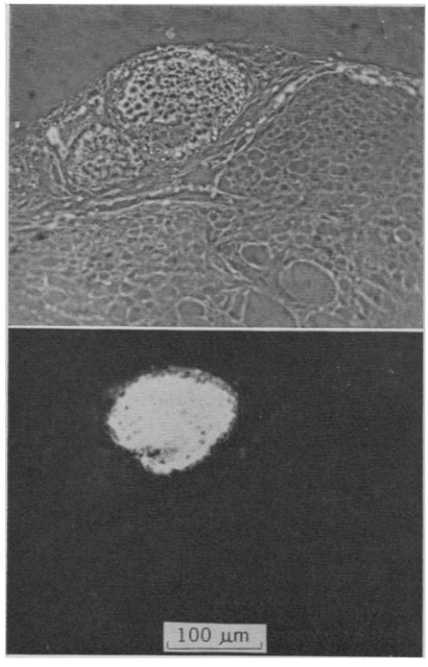


Fig. 1. The cell body of an injected neuron. (Top) A phase-contrast photograph of a 10-μm section through a ganglion in which a cell had been injected with Procion Yellow M4RS. (Bottom) The same section as seen in a fluorescence microscope. There is no spread of dye to neighboring neurons.

In lobster abdominal ganglia the cell bodies are arranged in characteristic groupings. The precise position of each individual cell within a group is not always the same, however, nor is the exact geometrical relationship between groups (*1*). Lobster central neurons are monopolar cells with their synaptic contacts deep in the neuropil. Since there are no synaptic contacts on cell bodies or the initial portion of the process entering the neuropil, it is possible that the variability in the final position of the cell body may be trivial, but the neuropil, where intercellular contacts are made, may be highly ordered.

No fully satisfactory method exists for tracing the course of individual neurons within the neuropil. Certain conventional neuroanatomical methods, such as staining with methylene blue or silver, will apparently stain cells in their entirety. However, the success of these methods depends on staining a random minority of nerve cells, and the probability of staining any particular cell is therefore quite low. A technique devised by Alvarez and Furshpan (*3*) seemed ideally suited to exploring the organization of the neuropil. The principle of their method is to inject dye into a cell through a microelectrode used for recording the physiological activity of the cell; after the dye has diffused into the tissue, the tissue is fixed, dehydrated, embedded, and sectioned serially so that dye-filled profiles can be traced.

We now describe our method of exploring the organization of the neuropil in lobster ganglia. Our major improvement in the method of Alvarez and Furshpan has been the selection of a class of dye with exteremly favorable properties. The results we present are selected to indicate the potentialities of the method, which we believe will have general applicability in studies where a knowledge of cellular geometry is important (*4*).

The selection of a dye which would diffuse well into the branches of an injected cell and survive histological procedures proved to be a major undertaking. After a series of studies with dyes that had been used by other investigators for intracellular injection (*5*), it became apparent that fluorescent dyes with reactive groups that bind covalently to macromolecules in the cell should be most useful, and Procion dyes (*6*) were the most successful. These are derivatives of cyanuric chloride which form covalent bonds with carbohydrates and proteins (*7*). Their detailed formulas are kept secret by the manufacturers. Fifty-eight Procion dyes and five related compounds, the Procion supra dyes, were screened, and in no case did we observe the extensive diffusion out of cells that had been seen with other dyes (Figs. 1 and 2).

The four best dyes were Procion Brown H3RS, Brilliant Red H3BNS, Navy Blue H3RS, and Yellow M4RS. Procion Yellow M4RS is more fluorescent than the other three, and we now use it exclusively. Its fluorescence is activated at 460 nm, and it emits at 550 nm. We have not studied the chemistry of the binding of dye to macromolecules in injected cells. The simplest assumption is that reactive groupings are directly alkylated, but we cannot eliminate the possibility that aldehyde fixatives have a role in the binding. Procion Yellow is fairly nontoxic to cells: 4 hours after injection of the dye we found no noticeable change in the resting potential, on the ability to conduct action potentials, or on synaptic potentials.

Cells in lobster abdominal ganglia were exposed and identified by previously published methods (*1*). Electrodes were filled with a 4 percent (weight to volume) solution of dye in distilled water; the electrode resistance with dye was about 10 megohms. Hyperpolarizing current pulses of $1 \times 10^{-8}$ to $5 \times 10^{-8}$ amp and 0.5 second long at a frequency of one per second were passed through electrodes until cells were well filled with dye, usually after about ½ hour. After injection the preparations were kept at 4°C for about 16 hours before fixation in aldehyde mixtures (usually overnight).

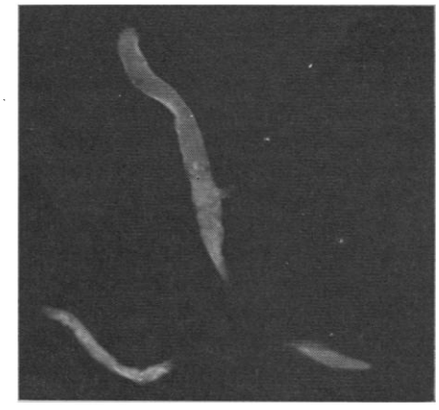

Fig. 2. Some branches of an injected neuron deep in the neuropil viewed in a fluorescence microscope. The scale is the same as in Fig. 1.

The principal effect of varying the aldehyde composition and *p*H of the fixative is on the intensity of a background green fluorescence of the histological sections. We routinely used a mixture of glutaraldehyde (6 percent) and acrolein (2 percent) at *p*H 4.0 as fixative. This gave a background which was easy to see, yet gave good contrast between dyed and undyed structures. Since the chemical basis of the background fluorescence of undyed tissue is not known, the effects of fixatives on the background in other tissues may be quite different from that observed in the lobster ganglion.

After fixation the tissue was dehydrated in a graded methanol series and embedded in Epon-812. Serial 10-μm sections were cut with a steel knife, mounted on slides in Lustrex, and examined for fluorescent dye profiles. Photographic enlargements of phase-contrast images (on semitransparent paper) of the serial sections were made, and the positions of dye-containing profiles were marked. The photographs were oriented relative to each other, and register marks were made. Projections of the profiles of the cell onto horizontal and vertical planes were constructed from the oriented photographs.

The horizontal projection of the result of one experiment with the inhibitory cell to the fast flexor muscles ($I_2$) is shown in Fig. 3A. In this experiment

it is likely that dye had pentetrated to the endings of the cell. Fine branches which covered the entire neuropil of the ganglion contained dye. There were several places where it was possible to follow dye down very fine profiles ($< 1\ \mu m$) which were well filled and ended abruptly as though nerve processes had terminated. Obviously, the validity of these surmises can be established only by electron microscopy. On examination of this view and the front view of the same cell (Fig. 3B), there is rather striking bilaterial symmetry of the destinations to which branches are sent in both sides of the neuropil, even though these points may be reached by different routes on the two sides. Some of the other cells in the ganglion also have a similar degree of bilateral symmetry of their branches in the neuropil, whereas still others (for example, the inhibitory cell to the slow flexor muscles, $I_1$) do not. We do not yet understand the significance of this internal symmetry, but, since the musculature on the two sides of the abdomen is innervated separately, shared input connections to pairs of efferent neurons may be another means, besides the electrical connections that are known to exist between certain bilateral pairs of cells (*1*), of ensuring that muscles contract in synchrony in the abdomen.

By injecting the same cell (for example, $I_2$) in a number of animals it has been possible to demonstrate that the outlines of the branching pattern of an individual cell was characteristic. There was, however, some microheterogeneity in the finer branches.

Through the analysis of regions in the neuropil to which particular cells sent their processes, a functional subdivision of the neuropil was begun. For example, there was a region receiving processes from the excitatory neurons of the fast flexor muscles and the inhibitory neuron of the fast extensor muscles, but none from the antagonistic set of neurons (the inhibitory neuron of the fast flexor muscles and the excitatory neurons of the fast extensor muscles); this region might contain interneurons concerned with fast flexion. Although conclusions such as these are of necessity very preliminary, they provide a valuable basis for a rational electrophysiological examination of the neuropil.

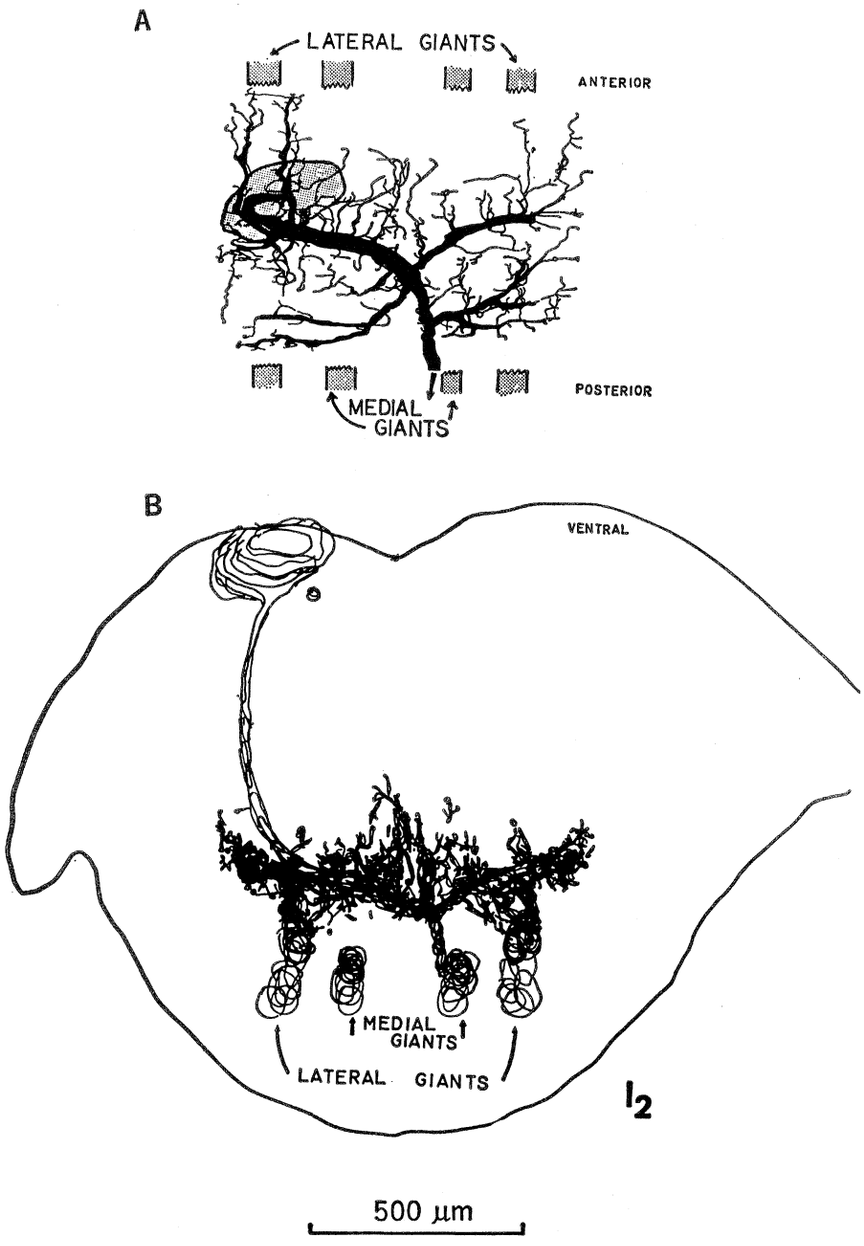


Fig. 3 (A) Plan (horizontal projection) of cell $I_2$ in the lobster second abdominal ganglion. The cell body is stippled; the axon continues in the direction of the arrow to the third root, where it leaves the ganglionic chain to innervate fast flexor muscles. The position of the medial and lateral giant fibers is indicated at the two ends of the ganglion. (B) Front projection of $I_2$. The outlines of the medial and lateral giant fibers have been drawn at 100-$\mu$m intervals.

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**References and Notes**

1. M. Otsuka, E. A. Kravitz, D. D. Potter, *J. Neurophysiol.* **30**, 725 (1967).
2. R. E. Coggeshall, *ibid.*, p. 1263; W. T. Frazier, I. Kupferman, R. E. Coggeshall, E. R. Kandel, R. Waziri, *ibid.*, p. 1288; E. R. Kandel, R. Waziri, W. T. Frazier, R. E. Coggeshall, *ibid.*, p. 1352; D. A. Baylor and J. G. Nicholls, in *Physiological and Biochemical Aspects of Nervous Integration*, F. D. Carlson, Ed. (Prentice-Hall, Englewood Cliffs, N.J., 1968), p. 3; M. J. Cohen and J. W. Jacklett, *Phil. Trans. Roy. Soc. London Ser. B* **252**, 561 (1967); A. O. D. Willows, *Science* **157**, 570 (1967); C. Rovainen, *J. Neurophysiol.* **30**, 1000, 1024 (1967).
3. J. Alvarez and E. J. Furshpan, unpublished results.
4. A. O. W. Stretton and E. A. Kravitz, in preparation.
5. R. C. Thomas and V. J. Wilson, *Nature* **206**, 211 (1965); *J. Neurophysiol.* **30**, 661 (1967).
6. Kindly supplied by Imperial Chemical Industries Organics, Inc., Providence, R.I., through the courtesy of Mr. Thornton.
7. R. Hess and A. G. E. Pearse, *Nature* **183**, 260 (1959); T. Vickerstaff, *Melliand Textilber* **30**, 905 (1958).
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