EVALUATION OF A RADIOAUTOGRAPHIC NEUROANATOMIC TRACING METHOD*

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INTRODUCTION

The availability of simple and reliable methods for establishing the precise distribution of specific fiber pathways within the central nervous system (CNS) has been a problem of continuing interest to the neurologist. Early neuroanatomical studies almost exclusively consisted of evaluations made in normal histological material. Subsequently, a number of more sophisticated tracing methods have been developed. Included among these are electrophysiological mapping technics, selective degeneration staining methods, and the tracing of degenerating pathways by means of electron microscopy. While each of these approaches has contributed greatly to our knowledge about fiber projections within the CNS, each of them is beset with certain limitations.

The purpose of the present study was to develop a neuroanatomical tracing method which would not be dependent on degeneration and which would be capable of reliably demonstrating the entire distribution of selected pathways within the CNS. The development of the radioautographic tracing method to be discussed is based on principles established chiefly within the past decade.

The existence of a proximodistal flow of axoplasm away from the cell body has been demonstrated by a variety of methods. A number of studies have shown that, following local injection of tritiated amino acid into the nervous system, the radioisotopic label is incorporated into protein within neuron cell bodies and is subsequently transported into the axons of these cells.

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Several studies have indicated that a portion of the incorporated label moves proximodistally within the axons at a velocity in the order of 100 mm/day\textsuperscript{18,19,25,41} and that some label ultimately passes into the terminal regions of the axons\textsuperscript{9,10,16,38}. Radioautographic investigations have demonstrated that, in regions remote from the site of injection, the label is confined exclusively to axons originating from cells whose somata lie in the vicinity of the injection\textsuperscript{18,28,38}.

These findings suggest that the local deposition of tritiated amino acids into the nervous system, coupled with subsequent radioautographic analysis, could possibly serve as a method for delineating the total course and distribution of selected fiber systems within the CNS. Such a method could potentially serve as a means for quantitatively evaluating fiber distribution density and for determining, by both light- and electron microscopic analyses, the precise projection of intact axonal systems.

**MATERIALS AND METHODS**

**Surgery and injections**

Adult cats were anesthetized by way of the intraperitoneal route with a solution of sodium pentobarbital (45 mg/kg body weight). The dorsal surface of the right L\textsubscript{7} spinal ganglion was exposed by means of a spinal laminectomy under aseptic conditions. The pelvis of the animal was stabilized in order to isolate the exposed ganglion from the cat's respiratory movements.

Three small injections of \textsuperscript{3}H-L-leucine were then made into different areas of the L\textsubscript{7} ganglion. The injection technic was similar to that previously described by Lasek\textsuperscript{18,19} and consisted of the following procedures. Each injection consisted of 1 \textmu l of normal saline containing 5 \textmu C of \textsuperscript{3}H-L-leucine (specific activity, 5C/mmole). A glass micropipette, 30-70 \mu in external diameter at the tip, was introduced into the ganglion to a depth of 0.4-0.8 mm below the ganglion's surface. The movement of the \textsuperscript{3}H-L-leucine solution into the ganglion was initiated by advancing the plunger of a 10 \textmu l Hamilton syringe which was connected to the micropipette by way of polyethylene tubing. Each injection was infused into the ganglion over a period of 5-10 min by mechanically controlling the rate at which the plunger of the \textmu l syringe advanced. The injections were carried out under observation through a binocular dissecting microscope. Following ganglion injection, the micropipette was removed, the subcutaneous tissues and skin were sutured closed, and the animal was given supportive care until it had recovered from anesthesia.

Toads (*Bufo marinus*) weighing between 275 and 325 g were also employed in this investigation. The toads were anesthetized by means of an intraperitoneal injection of a 2\% solution of MS-222 (0.8 ml/100 g body weight). The ninth spinal ganglion on the right side was surgically exposed, and \textsuperscript{3}H-L-leucine solution was then injected by a technic similar to that described above. Two injections of 0.5 \textmu l of normal saline containing 2.5 \textmu C of \textsuperscript{3}H-L-leucine were made into the exposed ganglion. Following ganglion injection, the wound was repaired and the animal was permitted to survive for a variable postoperative period. During this interval, the animal was kept in 20\degree C environment and was maintained in a well-nourished state.
Sampling of tissue

The cats were sacrificed at 12 h, 1 day and 7 days after injection. The toads were sacrificed at 1, 8, 10, 15, 20, 30 and 32 days after injection. The animals were anesthetized and were then sacrificed by perfusion through the heart with 10% formaldehyde or Bouin's fixative. When the perfusion in the cats was complete, the injected ganglion and the lumbar spinal cord were removed and placed in fixative. In the case of the toads, the injected ganglion and the entire CNS were removed and placed in fixative.

Radioautography

The tissues were fixed for approximately 1 week in either Bouin's fixative or 10% formaldehyde, and were then dehydrated and imbedded in paraffin. Serial transverse sections, 10 μ in thickness, were cut from the paraffin blocks, and were mounted on slides. The slides were then coated with Kodak NTB2 bulk emulsion according to the method of Kopriwa and Leblond and were stored in a darkened refrigerator for periods ranging from 4 weeks to 4 months. After development in Dektol, the sections were stained with Harris' hematoxylin. In all cases, the ganglia were processed before the CNS tissues. If a substantial number of ganglion cell bodies were found to be heavily labeled, then the CNS was processed according to the methods described above.

Of the 15 toads which were studied, only 5 were considered to have been adequately injected. In contrast, all 7 of the injected cats exhibited a high level of ganglion cell labeling. The relative lack of success in the injection of the toads was probably due to the very small size of the toad ganglion and the difficulty encountered in reliably introducing the micropipette into this small piece of tissue.

The distribution of silver grains within the radioautographs of the CNS of both cat and toad was assessed by quantitative, as well as subjective analysis. Counting of silver grains was performed under oil immersion with the aid of a Whipple ocular micrometer. The number of silver grains within the area circumscribed by the Whipple grid was determined for several regions of the CNS, on both the injected and uninjected sides. At the magnification employed (× 1000), the Whipple grid circumscribed an area of 6400 sq.μ on the tissue sections. In all cases where comparisons were made between grain counts in different regions, the length of emulsion exposure and the conditions of development were identical.

Comparison with Nauta-stained material

The total central distribution of the ninth spinal dorsal root of toad as defined by the radioautographic tracing method was compared with that previously delineated in our laboratory by the Nauta method. Also the segmental distribution of the L7 dorsal root of cat as defined by the radioautographic method was compared with the central projection of the cat's L7 dorsal root as delineated in our laboratory by the Nauta technic (unpublished observations). The degeneration-staining method employed in the above studies was the uranyl nitrate modification of the Nauta silver technic.

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RESULTS

Comparison of radioautographic and Nauta technics in toad

A comparison has been made between the central distribution of the ninth spinal dorsal root of toad (Bufo marinus) as delineated by the radioautographic tracing method and that previously defined by the Nauta method\textsuperscript{12}. Figs. 1 and 2 present the results obtained by the two methods.

Both the radioautographic and the Nauta tracing methods demonstrated that dorsal root fibers distribute unilaterally within the CNS. The greatest density of root distribution, as defined by each method, was at levels in and adjacent to the segment of root entrance (Figs. 1B, 1C, 1D). Both methods also showed that primary afferent fibers reached no further ventrally than the intermediate zone of the gray matter (Figs. 1C, 1D). Each method delineated a projection which passed to a dorsolateral region capping the dorsal horn (Figs. 1B, 1E). Similarly, each method defined a primary afferent projection which passed medially up the dorsal column (Figs. 1F, 2A, 2B) into the brain stem (Figs. 2C, 2D, 2E) and ultimately into the granular cell layer of the cerebellum (Fig. 2F). The longitudinal extensiveness of distribution within

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The spinal gray matter and within the dorsolateral tract was approximately equivalent in radioautographic preparations and in Nauta-stained material. However, a greater proportion of fibers appeared to project into the dorsolateral tract in the radioautographic series (Figs. 1B, 1E, 6C). Also, in radioautographic material, a small projection was noted into the gray matter underlying the dorsal column at the level of the obex (Fig. 2C), whereas such a projection was not apparent in the Nauta-stained series.

Quantitative evaluation in toad radioautographs

The distribution of radioactivity depicted in Figs. 1 and 2 was largely based on subjective interpretation of the material, such as is similarly employed in degeneration technics (for example, in the Nauta method). An attempt was made to analyze the distribution of radioactivity by more objective methods. This evaluation was carried out by making a comparative quantitative analysis of the density of radioactivity in selected regions within various levels of the toad nervous system. More specifically, grain counts were performed in the radioautographs over comparable areas on the sides ipsilateral and contralateral to the injected ganglion.

Fig. 3 indicates the approximate areas of the toad nervous system in which grain counting was performed. Fig. 3A depicts a section selected from a level near the root entrance zone and is approximately comparable to the level shown in Fig. 1C. Figs. 3B, 3C and 3D represent sections from levels which are, respectively, comparable to those shown in Figs. 2A, 2C and 2F. Table I presents the numerical results of the
Fig. 3. Schematic representation of the approximate areas in which grain counting was performed in toad in: A, level of root entrance; B, thoracic spinal cord; C, level immediately rostral to obex; D, cerebellum. Dark squares, areas counted on the side of the injection; open squares, areas counted on the uninjected side. The numbers related to the squares define the areas of grain counting as they are listed in Table I.

above counting analysis. In several instances, the size of the area counted was varied in a manner which would assure that particular regions containing a sparse and/or diffuse distribution of fibers (e.g., Figs. 2C, F) would be reliably represented.

At the level of the root entrance zone (Fig. 3A), the densest activity was noted in the dorsolateral region of the ipsilateral gray matter (area 2). There was a five-fold difference between ipsilateral and contralateral sides with respect to the density of activity in this region (Table I). Counts significantly above background were also obtained in more medial and ventral portions of the ipsilateral dorsal horn (Fig. 3A, areas 1 and 3), while no appreciable difference was noted between ipsilateral and contralateral sides in the dorsal aspect of the ventral horn (Fig. 3A, areas 4 and 5).

At thoracic levels (Fig. 3B), there was a significant difference between the counts performed on either side of the cord in the dorsal portion of the dorsal column (Fig. 3B, area 1)—the activity of the ipsilateral side was approximately double that on the contralateral side. The absence of a significant difference between more ventral aspects of the dorsal column (Fig. 3B, area 2) may be related to the fact that approximately one-half the area counted was comprised of gray matter. At the level of the obex (Fig. 3C), ipsilateral counts in the dorsal column proper (Fig. 3C, area 1) and in the
TABLE I

THE NUMBER OF SILVER GRAINS PRESENT IN SELECTED REGIONS OF THE TOAD CNS

Each of grain counts presented in the table represents the mean and the standard deviation of the number of grains which were found within a comparable area in five adjacent tissue sections. The specific areas which were counted are denoted by their number in Fig. 3. This preparation was sacrificed 15 days after injection of \([\text{PH}]-\text{L}-\text{leucine}\).

<table>
<thead>
<tr>
<th>Area analyzed*</th>
<th>Injected side (grains (6400 \text{ sq. } \mu))</th>
<th>Unjected side (grains (6400 \text{ sq. } \mu))</th>
<th>Size of area counted in each section (sq. (\mu))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lumbar spinal cord</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3A, 1</td>
<td>112.2 ± 18.0</td>
<td>53.4 ± 12.4</td>
<td>6,400</td>
</tr>
<tr>
<td>3A, 2</td>
<td>787.2 ± 48.0</td>
<td>51.0 ± 14.3</td>
<td>6,400</td>
</tr>
<tr>
<td>3A, 3</td>
<td>79.4 ± 14.9</td>
<td>50.8 ± 6.5</td>
<td>6,400</td>
</tr>
<tr>
<td>3A, 4</td>
<td>53.2 ± 13.7</td>
<td>50.0 ± 6.9</td>
<td>6,400</td>
</tr>
<tr>
<td>3A, 5</td>
<td>59.4 ± 8.4</td>
<td>50.0 ± 5.6</td>
<td>6,400</td>
</tr>
<tr>
<td>Thoracic spinal cord</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3B, 1</td>
<td>116.6 ± 10.6</td>
<td>66.2 ± 15.7</td>
<td>6,400</td>
</tr>
<tr>
<td>3B, 2</td>
<td>76.4 ± 15.7</td>
<td>60.2 ± 10.9</td>
<td>6,400</td>
</tr>
<tr>
<td>Medulla oblongata</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3C, 1</td>
<td>150.5 ± 11.1</td>
<td>38.3 ± 2.9</td>
<td>12,800</td>
</tr>
<tr>
<td>3C, 2</td>
<td>85.0 ± 17.3</td>
<td>37.9 ± 3.3</td>
<td>12,800</td>
</tr>
<tr>
<td>Cerebellum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3D, 1</td>
<td>59.6 ± 7.9</td>
<td>37.1 ± 8.0</td>
<td>25,600</td>
</tr>
</tbody>
</table>

* The numbers represent areas depicted in Fig. 3.

In radioautographs of the cerebellum, the distribution of radioactivity was aggregated into relatively dense but spatially separated loci. The counts made in the cerebellum necessarily included a substantial area into which primary afferent fibers did not subjectively appear to distribute. Despite this circumstance, a significant difference in grain density was recorded between ipsilateral and contralateral sides of the cerebellum (Fig. 3D, area 1).

It should be noted that the variation in the number of grains found at different levels of the CNS on the unjected side (Table I) was merely due to variations in the level of background activity in the radioautographs. This fact was ascertained by making grain counts on areas of the slides devoid of tissue section. The counts in these areas were found to closely correspond to that in the contralateral half of the nervous system.

Comparison of radioautographic and Nauta technics in cat

The segmental distribution of primary afferent fibers within the lumbar cord of a cat sacrificed 7 days following injection corresponded, in general, with that reported in previous studies employing the Nauta method and with that recorded in studies in our own laboratory employing the Nauta method (unpublished observations). Thus, label was distributed over the ipsilateral dorsal column, inter-

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mediate gray matter and ventral horn, and most heavily in the ipsilateral dorsal horn. The distribution appeared to be entirely ipsilateral to the side of the injection. For example, an abundant number of grains were observed in the dorsal horn ipsilateral to the injection (Fig. 5D), while virtually none were apparent on the contralateral side (Fig. 5C).

The only major difference between the cat dorsal root distribution delineated by the radioautographic technic and by the Nauta method was the fact that the radioautographs showed a considerable primary afferent projection to the substantia gelatinosa (lamina II of Rexed) which was not as apparent in Nauta-stained material (Figs. 7A, 7B).

**Quantitative evaluation in cat radioautographs**

Grain counts were performed in the cat material in order to quantitatively evaluate the distribution which was subjectively noted. Fig. 4 illustrates the approximate location of the 10 areas counted. As in the toad material, counts were performed in comparable areas in both halves of the spinal cord. Table II presents the results of the above analysis. The level of activity in the ipsilateral dorsal horn of the cat was substantially above that recorded in the contralateral dorsal horn (areas 5, 6, 7), as was similarly true for the dorsal column (area 1). Significant differences, but of a lesser order of magnitude, were also noted between the ipsilateral and the contralateral ventral gray matter (areas 8, 9, 10). The level of activity in the ipsilateral ventral and lateral white matter (areas 2, 3, 4) was equivalent to that recorded in all areas of the uninjected side and in areas of the emulsion which did not contain subjacent tissue.

**Time factor**

The distribution of label just discussed was recorded in a cat which had been sacrificed 7 days following operation. Another cat sacrificed after a 7 day post-injection interval exhibited a distribution of label practically identical to that already

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**Fig. 4.** Schematic representation of the approximate areas in which grain counting was performed in the L7 segment of cat spinal cord. The darkened squares indicate the areas counted on the injected side. Comparable areas were counted on the contralateral side of the spinal cord. The number beside each square defines the area counted as it is listed in Table II.
TABLE II

THE NUMBER OF SILVER GRAINS PRESENT IN SELECTED REGIONS OF THE L7 SEGMENT OF CAT SPINAL CORD

Each of the grain counts presented in the table represents the mean and standard deviation of the number of grains which were found within a comparable 6400 sq. μ area in five adjacent tissue sections. The same five sections were employed for all counts. The specific areas which were counted are denoted by their number in Fig. 4. This preparation was sacrificed 7 days after injection of \([^{3}H]-L\)-leucine.

<table>
<thead>
<tr>
<th>Area analyzed*</th>
<th>Injected side (grains/6400 sq. μ)</th>
<th>Uninjected side (grains/6400 sq. μ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Post. white col.</td>
<td>157.5 ± 16.4</td>
<td>25.5 ± 8.1</td>
</tr>
<tr>
<td>2 Lat. white col.</td>
<td>29.3 ± 7.3</td>
<td>24.2 ± 5.1</td>
</tr>
<tr>
<td>3 Lat. white col.</td>
<td>26.4 ± 6.3</td>
<td>23.9 ± 5.6</td>
</tr>
<tr>
<td>4 Vent. white col.</td>
<td>23.3 ± 6.4</td>
<td>24.0 ± 7.0</td>
</tr>
<tr>
<td>5 Dorsal horn</td>
<td>more than 500**</td>
<td>25.7 ± 4.4</td>
</tr>
<tr>
<td>6 Dorsal horn</td>
<td>145.6 ± 10.2</td>
<td>21.0 ± 5.4</td>
</tr>
<tr>
<td>7 Dorsal horn</td>
<td>119.1 ± 12.7</td>
<td>27.7 ± 0.9</td>
</tr>
<tr>
<td>8 Ventral horn</td>
<td>44.5 ± 11.5</td>
<td>20.1 ± 2.9</td>
</tr>
<tr>
<td>9 Ventral horn</td>
<td>47.3 ± 9.2</td>
<td>32.3 ± 2.0</td>
</tr>
<tr>
<td>10 Ventral horn</td>
<td>49.0 ± 5.5</td>
<td>28.3 ± 3.4</td>
</tr>
</tbody>
</table>

* The numbers represent areas depicted in Fig. 4.
** The distribution of label in this area on the injected side was so dense that accurate grain counts could not be made.

discussed. Five cats which had been sacrificed at 1 day or less following injection exhibited small amounts of label in the ipsilateral dorsal horn, but no distinguishable label in the ventral horn. It should also be noted that a subtotal pattern of distribution was noted in a toad which had been sacrificed after a 1-day postinjection interval.

**Question of extracellular spread of label**

The possibility that a substantial amount of label noted in the spinal cord could have arrived there by way of extracellular spaces was investigated. In the ganglia, label was localized in the vicinity of the injection (Fig. 5A). In the dorsal root (Fig. 6D) and in the dorsal column (Fig. 6B), label was exclusively confined to the axon proper. It has already been noted that the label was unilaterally distributed within the spinal cord. The walls of blood vessels, which were located within regions of dense activity, did not contain label (Fig. 5B). Label was not uniformly distributed within the dorsal quadrant of the ipsilateral spinal cord. The most impressive example of this non-uniformity was given by the counts in areas 2 and 5 of Fig. 4 and Table II. These grain counts were performed on either side of the division between gray and white matter forming the lateral border of the cat dorsal horn. Activity within area 5 was so dense that it was difficult to accurately count. In contrast, area 2 in the immediately adjacent white matter exhibited a level of activity which did not exceed background levels.

**Analytical limitations**

Although individual labeled fibers could be discerned in the white matter (Fig.
6B), it was usually not possible to identify individual axons within the gray matter in radioautographic preparations. In some instances, however, the aggregation of grains within the gray matter was such as to suggest small underlying groups of fibers running in parallel (Fig. 6A). In several instances, grains appeared to lie on the surface of dendrites and somata of large ventral horn cells in cat, but, again, these grains could not be related to particular neuronal structures.

DISCUSSION

In our present study, the radioautographic method has delineated dorsal root ganglion projections to the CNS which are generally comparable to that defined by other tracing methods, in particular the Nauta method. It has also been demonstrated that the radioautographic method may serve as a useful tool for statistically validating projections which are of questionable existence in subjective evaluations. These results indicate that the radioautographic tracing method can be employed as a reliable means for delineating the total distribution of particular fiber systems within the CNS.

The present study, along with others\textsuperscript{19,28,38}, has demonstrated that there is no significant spread of label by way of extracellular spaces to sites distant from that of a small local injection of label into the nervous system. The circumscribed labeling of cell bodies within the injected ganglia, and the confinement of label to regions of the axon proper in the dorsal root and the dorsal column suggest that there was no significant spread of label by way of the perineural connective tissue spaces. The absence of label (above background levels) in the uninjected half of the spinal cord, particularly at the dorsal periphery, suggest that there was no significant spread of label by way of the cerebrospinal fluid space. Finally, the marked non-uniformity of labeling in the dorsal quadrant of the spinal cord on the injected side, coupled with the lack of label in the walls of blood vessels lying in the vicinity of dense amounts of stromal labeling, suggests that there was no appreciable spread of label by way of the vascular system.

Therefore, it would appear that the entire distribution of label noted at sites distant from the local injection was solely a result of proximodistal streaming of label within axons which were extensions of cell bodies located in the vicinity of the injection. The great majority of the tritiated leucine which was recorded in our radioautographs was presumably located in protein fractions of the axoplasm which were originally manufactured in the cell body and which were then subsequently conveyed somatofugally within the axon\textsuperscript{2,5,28}. In this regard, it should be noted that some studies have shown that axons may pick up tritiated amino acids locally from the surrounding tissue environment\textsuperscript{6,14,32}. However, the relative amount of labeled

Fig. 5. A, L7 dorsal root ganglion and ventral root from a cat sacrificed 1 day after injection of label illustrates labeled ganglion cells (LC). (× 55). B, L7 segment of cat spinal cord in the region of the apex of the dorsal horn illustrates unlabeled blood vessel (bv) and unlabeled pia mater (arrows). (× 880). C, Apex of dorsal horn of L7 segment in cat on the uninjected side. (× 700). D, Apex of dorsal horn of L7 segment on the injected side in same animal shown in C. (× 700).

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amino acid which is received in this manner is so small that from a practical standpoint such label is not capable of appreciably labeling portions of axons lying distant from the injection site\textsuperscript{5,10}.

The distribution of label into areas of the toad nervous system in which primary afferent fibers have been previously shown to terminate in this form\textsuperscript{12} gives further support to the contention that incorporated label is carried within the axoplasm all the way from the cell body to terminal regions of the axons\textsuperscript{10,16,38}. It is not presently known with certainty whether or not such transported label will subsequently pass into postsynaptic units.

It has been noted that the radioautographs demonstrated heavy labeling of the dorsolateral tract in toad, dense labeling of the substantia gelatinosa in cat, and definite labeling of the brain stem gray matter underlying the dorsal column at the level of the obex in toad. In contrast, primary afferent projections to the above areas appeared to be less dense or non-existent in Nauta-stained material. The dorsal root projection into the dorsolateral tract of amphibians is almost exclusively composed of small diameter fibers\textsuperscript{3,12}. Laminae I–III of the cat dorsal horn contain a substantial number of small diameter primary afferent fibers\textsuperscript{3,38}. However, the extent of the projection of primary afferent fibers into the cat substantia gelatinosa (lamina II of Rexed) has been minimized in certain previous degeneration studies\textsuperscript{29,30}. The primary afferent projection to the brain stem gray matter in toad, delineated by the radioautographic method in the present study, probably represents a terminal field within the primitive dorsal column nucleus described in amphibians\textsuperscript{39}. This field would presumably be characterized by preterminal-terminal extensions of small caliber. These facts suggest that the radioautographic method may be a better means for defining the projections of small diameter fibers than the Nauta method.

It should be noted that we have studied the merit of the radioautographic tracing method in a relatively unique subdivision of the nervous system. Our selection of a primary afferent system with cell bodies lying extraneous to the CNS proper does not permit us to declare with certainty that the radioautographic method is also applicable to fiber systems which are totally intrinsic to the CNS. However, the fact that label injected into relatively small areas of the cat ganglion was primarily incorporated by cells in the immediate vicinity of the injection suggests that injection of label into highly localized areas within the CNS would also be possible.

If radioautographic tracing method did prove to be applicable to the tracing of fiber systems intrinsic to the CNS, this technic would offer certain striking advantages over degeneration methods. For example, the lesions employed in degeneration methods usually do not have the capacity to selectively destroy only the cell bodies lying in particular areas of the CNS. Fibers passing through these particular regions are also inevitably destroyed. The radioautographic tracing method would

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Fig. 6. A, Intermediate gray matter of cat L\textsubscript{7} segment illustrates a labeled bundle of axons cut tangentially (arrows) and an unlabeled bundle of fibers (U). (× 880). B, Cat lumbar dorsal column illustrates labeled axon (arrows). (× 880). C, Toad dorsolateral zone on injected side at a level two segments rostral to the root entrance zone of labeled ganglion cells. The lateral edge of the cord is at the top of the figure. (× 880). D, L\textsubscript{7} dorsal rootlet from cat illustrates labeled axons (arrows). (× 700).

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Fig. 7. A, Dorsal horn of L7 spinal segment in cat stained with the Nauta technique. (× 80). B, Radiographic appearance of an area comparable to that defined by the rectangular outline in A. (× 500).
theoretically permit a labeling of only cell bodies within the site of injection leaving axons passing through such sites unappreciably altered. In addition, the radioautographic tracing method would permit one to positively ascertain which particular cell bodies were the source of origin for the axonal pathways delineated. Similar evaluations are not possible in degeneration studies where the entire area of origin for the axons in question has been obliterated by the lesion imposed.

Some of the limitations of the radioautographic tracing method, as defined in the present study, are outlined below. The portion of the nervous system to be injected must be of an adequate size and consistency and in a suitable locus to permit placement of a glass micropipette. The analysis of fiber distribution is not as easily accomplished in radioautographic material as it is in Nauta-stained material. In the analysis of Nauta-stained material one could employ relatively low microscopic magnification and could often discern the course of individual fibers over relatively long distances. On the other hand, relatively high microscopic magnifications were required to accurately locate grains in radioautographs and only rarely were grains so heavily distributed along individual axons that specific fibers stood out as discrete entities within the gray matter. Furthermore, because the stains normally employed with radioautography do not provide adequate neurohistological detail in the CNS, it was often difficult to relate particular grains to particular axonal or terminal processes. The majority of limitations are technical ones which are intrinsic to the method of radioautography. For example, there are several weeks or months of waiting for the emulsion to become adequately exposed and there is often an appreciable level of background activity (artefact) within radioautographs which have been exposed for such long intervals.

One of the principal technical problems encountered in degeneration tracing studies is the determination of the survival interval in which degeneration is maximally apparent along the entire extent of transected axons. A similar problem is encountered in radioautographic mapping, for it is of critical importance to determine the postinjection interval in which label is maximally distributed along the entire length of the axons in question. Our results in toads maintained at 20°C and in cats suggest that a postinjection interval of 1–2 weeks is required to obtain such a distribution of label. This suggestion is indirectly supported by the fact that axons are maximally labeled by proximodistal axoplasmic streaming after a period of 6–10 days has elapsed following injection. However, a more extensive evaluation of this particular question is needed. One would anticipate that the optimum length of the postinjection interval would be dependent on the specific metabolic character of the neuronal system analyzed, on the length and diameter of the axons, on the CNS temperature, and so forth. Improvements in the radioautographic tracing method are in a sense largely dependent on the acquisition of further knowledge about the basic mechanics of axoplasmic streaming in general.

Despite the above limitations and uncertainties, the radioautographic tracing method has great potential as a neuroanatomical tool. It should be noted that this method is based on physiological principles very different from those which serve as the basis for electrophysiological mapping and for degeneration tracing methods.
In this sense, it serves as an entirely new approach to the analysis of neural fiber projections. Employment of the radioautographic tracing method would permit analysis of the distribution of selected intact axons at both light and electron microscopic levels. For example, the gross distribution of a selected fiber system could be delineated at the light microscopic level such as has been done in the present study. This investigation could be followed by an analysis of the normal terminal properties and relationships of that particular fiber system as defined in electron microscopic radioautographs. The authors are currently exploring this possibility.

SUMMARY

Local injections of \[^{3}H\]-L-leucine solution were made unilaterally into the ninth spinal ganglion of toads (Bufo marinus) and into the L7 dorsal root ganglion of cats. Injected animals were sacrificed at various postinjection intervals and their CNSs were subjected to radioautographic analysis. The dorsal root distributions delineated by the radioautographic tracing method were found to correspond closely with dorsal root projections defined by the Nauta method. However, the radioautographic technic appeared to define more reliably the distribution of small diameter fibers. In addition, the radioautographic method may be employed to quantitatively evaluate the validity of pathways which have been subjectively noted. Evidence was obtained which suggested that there was no significant spread of label from the site of injection to the CNS by way of extracellular spaces. The limitations and potential applications of the radioautographic tracing method are discussed.

ADDENDUM

Since this paper was submitted for publication, a report by Goldberg and Kotani has appeared in which autoradiography has been used to trace frog optic nerve fibers\[^{3}H\]. The results of their paper are in agreement with the study reported here.

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