Technical Note

Retrograde Tracing of Zinc-containing Neurons by Selenide Ions: A Survey of Seven Selenium Compounds

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The autometallographic retrograde tracing of zinc-containing neurons by intracerebral injection of sodium selenite (Na2SeO3), introduced by Danscher in 1982, has recently been described in more detail. Intracerebral injections of both sodium selenide (Na2Se) and sodium selenite (Na2SeO3) have been successfully used; however, sodium selenide had a rather toxic effect on the injected tissue. In the present study, we tested seven different selenium compounds to find the most suitable compound for retrograde tracing of zinc-positive pathways. Among the tested compounds, sodium selenide (Na2Se) caused insignificant necrosis within the injection site and was easily transported retrogradely when handled anaerobically. Sodium selenide is therefore recommended as the compound of choice. (J Histochem Cytochem 40:575-579, 1992)

KEY WORDS: Zinc; Zinc-containing neurons; Selenium; Toxocity; Retrograde tracing; Autometallography; Rat.

Introduction

The histochemically reactive pool of zinc in the brain is almost exclusively restricted to the neuropil (3,6,17), where it is localized to the boutons (3,6,17,18) and, more precisely, within the vesicles (3,15,21,24,25). Neurons giving rise to these zinc-containing boutons are called zinc-containing neurons. The vesicular zinc pool can be demonstrated histochemically by four methods: the dithizone (12), quinoline fluorescence (14), Neo-Timm (7), and the selenium method (6). The two former methods are based on chelation, whereas the Neo-Timm and the selenium methods are based on autometallographic silver enhancement of zinc bound in situ, either as zinc sulfide or zinc selenide (4,6,8,14,22).

Until it was observed that intrahippocampal injection of sodium selenite resulted in the appearance of what was believed to be zinc selenide accumulations in somata of the contralateral hippocampus (2,6), the only tools for mapping zinc-containing pathways have been traditional lesion-degeneration methods combined with the Neo-Timm or the selenium method (13,26,27).

As sodium selenide (Na2SeO3) was found to have a rather toxic effect on the injected brain tissue (2,3,6), sodium selenide (Na2Se) was suggested as a tracer substance for local application (2). It was found that after autometallographic silver enhancement most or all of the retrogradely transported zinc selenide was located in lysosome-like organelles (2). These results have recently been carefully tested and the technique for chemospecific retrograde tracing of zinc-containing neurons in the brain has been refined (18).

In rats subjected to intraperitoneal injection of sodium selenite and with a survival time of 24 hr, Slomianka et al. (28) have demonstrated that probably all neurons of origin of zinc-containing terminals are labeled by retrograde transport of zinc selenide. The original selenium method causes binding of all vesicular zinc in zinc-containing boutons (7). The observed loading of all zinc-containing somata is therefore the result of a retrograde transport of zinc selenide in all zinc-containing neurons (28).

The basic principle of the local tracing method is that the selenium anions, when infused into the brain, form zinc selenide in the synaptic vesicles of zinc-containing neurons. The zinc selenide precipitate, in turn, is transported retrogradely to neural perikarya, where it seems to accumulate in the lysosomes (2,18) and subsequently can be rendered visible by autometallographic silver enhancement. Silver grains demonstrating the presence of zinc selenide are visible in light and electron microscopy (2,3,6,8,18,28).

Prior work has shown that either the Se2- or the SeO32- ion can be used for the retrograde labeling procedure (2,6,18,19), with the Se2- ion yielding somewhat less necrosis at the infusion site (2,3,18).

The present work was undertaken to further investigate and potentially improve on the existing selenium method of retrograde labeling. At issue was whether different selenium-containing molecules would produce different results in retrograde labeling; in all, seven different selenium-containing compounds were tested.

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Several specific practical issues were explored, i.e., (a) given the difficulties in obtaining and maintaining the (unoxidized) \( \text{Na}_2\text{Se} \) compound, might a more convenient molecule be used instead? (b) Might a different compound yield greater amounts of retrograde labeling, i.e., a higher sensitivity than the \( \text{Na}_2\text{Se}/\text{Na}_2\text{SeO}_3 \) method, and (c) do all selenium compounds produce some tissue damage? In addition, as a matter of theoretical interest in the interpretation of the retrograde data, we tested the assumption that retrograde labeling of neurons could only be produced with selenium compounds that produced clear labeling of the axonal boutons (i.e., formation of zinc selenide) at the injection site.

Our findings indicate that \( \text{Na}_2\text{Se} \) is the best available molecule for retrograde labeling, and that retrograde labeling of perikarya is found only with selenium compounds that yield labeling of the axonal boutons at the injection site.

**Materials and Methods**

Adult male Wistar and Sprague-Dawley rats from 200–450 g were used. Material from over 50 rats was reviewed for this report.

The following chemicals were tested:

1. Sodium selenite (\( \text{Na}_2\text{SeO}_3 \)) (Sigma; St Louis, MO).
2. Sodium selenide (\( \text{Na}_2\text{Se} \)) (Ventron; Karlsruhe, FRG).
3. Selenium tetrachloride (\( \text{SeCl}_4 \)) (Merck; Darmstadt, FRG).
4. Sodium selenate (\( \text{Na}_2\text{SeO}_4 \)) (BDH Chemicals; Poole, UK).
5. Selenomethionine (Sigma).
6. Powdered selenium (Struers Chemical Lab; Rodovre, Denmark).
7. Dimethyl selenium (\( (\text{CH}_3)_2\text{Se} \)) (Sigma).

Solutions (0.2%w/v) of sodium selenite (\( \text{Na}_2\text{SeO}_3 \)) and equimolar solutions of the remaining compounds were used. Sodium selenite (\( \text{Na}_2\text{SeO}_3 \)), sodium selenide (\( \text{Na}_2\text{Se} \)), sodium selenate (\( \text{Na}_2\text{SeO}_4 \)), selenium tetrachloride (\( \text{SeCl}_4 \)), and selenomethionine were dissolved in a deoxygenated 0.1 M Sørensen’s phosphate buffer and the solutions were kept in an air-tight box. Dimethyl selenide was dissolved in glycerol.

**Pressure Injections.** The animals were anesthetized with pentobarbital. Bilateral stereotaxic injections were performed into the dorsal striatum (coordinates: anterior 0.5 mm, lateral 3.0 mm, and ventral –5.5 mm) (23). Of each compound, 0.02 µl was injected over a 5-min period, using a Hamilton microsyringe. After the injection, the syringe was left in situ for another 5 min.

Powdered selenium was introduced by tapping into the tip of a glass micropipette with a tip diameter of 40–50 µm. Pipettes were implanted bilaterally at the above-mentioned coordinates and cemented onto the skull.

**Iontophoretic Application.** Under anesthesia, the neostriatum or the amygdaloid perirhinal cortical nucleus (coordinates: anterior –4.3 mm, lateral 4.0 mm, and ventral –9.6 mm) was reached with a glass micropipette (tip diameter 2–4 µm) filled with sodium selenide (\( \text{Na}_2\text{Se} \)) or sodium selenite (\( \text{Na}_2\text{SeO}_3 \)) and a current of 4 nA was delivered intermittently for a total of 25 sec over a 5-min period. After the application the micropipette was left in situ for another 5 min.

After 24 hr of survival, the animals were re-anesthetized and decapitated. The brain was quickly removed from the skull and frozen with CO2.

**Results**

**Sodium Selenide, Sodium Selenite, and Selenium Tetrachloride**

Of the seven compounds that were tested, only three produced any consistent staining of the neuropil at the injection site. These three were sodium selenite, sodium selenide, and selenium tetrachloride. After pressure injections of a given volume of solution, the sizes of the selenite- and sodium selenide-labeled zones were comparable, whereas the apparent injection site produced by the selenium tetrachloride was smaller (Figures 1a and 2a). All pressure injections produced a necrotic zone in the center of the injection site (Figures 1a and 2a), but among the three compounds that did produce labeling, the sodium selenide produced the least apparent damage at the injection site. Within the central necrotic zone were found dead or dying neurons with shrunken perikarya, pyknotic nuclei, and hypochromatic nucleoli. These injured neurons often showed silver staining within the perikarya. Except for the occasional staining of the injured neurons, there was no other staining at injection sites that could not be interpreted as the staining of axonal boutons. Specifically, there was a consistent absence of staining in the white matter and in the perikarya of intact cells. All three compounds did produce robust retrograde labeling. Injection of sodium selenide and sodium selenite produced equal amounts of retrogradely labeled neuronal somata, whereas injection of selenium tetrachloride produced approximately 75% the amount of labeling of the former compounds.

Iontophoretic infusions of selenium were performed using the selenide and the sodium selenite compounds. As with the pressure injections, the results for the two compounds were comparable after iontophoresis. However, the destruction of tissue appeared to be consistently less with the sodium selenide compound. Selenide infusions into the striatum or the amygdaloid complex produced very discrete injection sites with little or no apparent neuron death (Figures 3a and 3b), and such injections produced robust retrograde labeling among zinc-containing neurons afferent to the injection site (Figures 4 and 5).
Sodium Selenate, Selenomethionine, Selenium, and Dimethyl Selenium

Except for an extremely faint trace of staining that was visible in darkfield illumination after selenomethionine injection, none of these four compounds produced any labeling in the vicinity of the injection site. Likewise, none of these compounds produced any retrograde labeling in the somata of neurons afferent to the injection site.

Discussion

The present study led to essentially three results: (a) some selenium compounds produce neuropil staining at an intracerebral injection site and some do not; (b) only those compounds that stain the neuropil yield retrograde labeling; and (c) of those that stain, the sodium selenide appears to produce the least amount of tissue damage.

Presumably, the staining of neuropil around the intracerebral infusion site is determined by the metabolic fate of the Se$^{2-}$ ions.
within the molecule. Therefore, the solution of Na₂Se would be expected to provide Se²⁻ which could enter vesicles and combine directly with Zn²⁺ to form the catalytic precipitate ZnSe. Metal atoms, such as zinc, are capable of stripping selenium from organic selenium compounds (29). A comparable reaction might be expected to take place in the boutons after local injection of selenium compounds. With Na₂SeO₃ and SeCl₄, some reduction by one or several endogenous reducing agents is required to convert the Se cations to Se anions, which can then react to form ZnSe.

As retrograde transport of zinc selenide takes place also after IP injections of sodium selenite (28), it can be deduced that it is important for retrograde axonal transport of ZnSe that the zinc-positive boutons are intact. It is our impression that iontophoretic application of selenide ions is superior for labeling zinc-positive somata, but we have not conducted qualitative studies on this. If, however, boutons with zinc-containing vesicles are injured, the possibility of axonal transport of zinc selenide accumulations is not to be expected (see below). Injurious alterations to the brain have not been observed after IP administration of sodium selenite, presumably because the sodium selenite is reduced in the peritoneal cavity or in the blood and is transported to the brain as selenide ions (2,7,28).

The three compounds, sodium selenide, sodium selenite, and selenium tetrachloride, all yielded somewhat equal amounts of retrograde labeling. Regardless of why many of the selenium compounds failed to stain the neuropil, it is significant that the retrograde labeling was found only in those compounds that produced neuropil staining at the injection site. This finding supports our prior assertion that retrograde labeling occurs by the retrograde transport of ZnSe from boutons to perikarya. According to this interpretation, selenium compounds that do not lead to the formation of ZnSe precipitate in the local boutons of the neuropil (i.e., produce no injection site staining) produce no retrograde labeling because they yield no catalytic reaction product (no ZnSe) that could be transported to perikarya (7). In this context it should be recalled that previous lesion studies (9,18,20) have yielded complementary results concerning the specificity of the retrograde method as a marker for zinc-containing neurons. Specifically, it has been shown in several brain areas that the retrograde labeling after selenium infusions occurs only in anatomic pathways of which the transection causes (in lesion experiments) the loss of zinc-containing axonal boutons in the target area (26). Therefore, this retrograde transport procedure depends on the presence of zinc-containing axonal boutons to supply the Zn²⁺ for the formation of the ZnSe catalyst in situ.

Because the selenium retrograde method is fundamentally different in biochemistry and physiology from conventional nonspecific retrograde methods, such as HRP and Fluoro-Gold, there are some special considerations. Mechanical or metabolic damage to the tissue, for example, does not promote retrograde labeling of zinc-containing neurons by the selenium method. Indeed, direct attempts to label zinc-containing cortical neurons by injections into the corpus callosum have failed (18), presumably because neither the cut nor the intact axons at the injection site contained appreciable amounts of Zn²⁺ that could form the ZnSe precipitate required for the retrograde labeling. Furthermore, because the pool of zinc that is labeled in boutons is initially in the vesicles, any cell damage that depletes this vesicular pool (10,11) before the formation of ZnSe can potentially prevent the retrograde labeling of the parent neuron. To label all zinc-positive neurons that terminate in a certain area of origin, it is crucial to keep the number of injured boutons as low as possible. This is particularly important when methods based on retrograde transport are used, especially in the study of projections with only a few terminals within the area of injection. The use of micropipettes and iontophoretic application substantially reduces the risk of injuring the studied pathway. Fi-
nally, it has been observed that dying cells develop an anomalous staining for zinc (10,11,16). The appearance of “pseudolabeled” neurons (18; and the present results) makes the search for labeled intact cells more difficult. For these reasons, a retrograde labeling method that causes minimal tissue damage is desirable.

In conclusion, sodium selenide has no deleterious effect on the tissue and is therefore recommended as the most suitable selenium compound for retrograde tracing of zinc-containing neurons.

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Literature Cited