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# The dithizone, Timm's sulphide silver and the selenium methods demonstrate a chelatable pool of zinc in CNS

## A proton activation (PIXE) analysis of carbon tetrachloride extracts from rat brains and spinal cords intravitally treated with dithizone

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**Summary.** From rats intravitally treated with dithizone (diphenyl-thiocarbazone) brains and spinal cords were removed and freeze-dried. The dithizonates present in the CNS tissue were extracted with carbon tetrachloride and subjected to a multielement analysis (proton activation, PIXE). It was found that the extract contained two metals. Most of the metal was zinc, but small traces of copper were also detected. Because prior treatment with the chelating agent, dithizone, can block both the Timm and the selenium metal staining methods, it is suggested that the three techniques label predominantly zinc in the neuropil (DTS-zinc).

### Introduction

It has been found that dithizone is an intravital marker of zinc in pancreas, eye, prostate glands and intestine (Stampfl 1959). When Maske (1955) used spectrophotometry to analyze carbon tetrachloride extracts from hippocampi of rats that had been treated intraperitoneally with dithizone, he found that the extracts contained zinc, as later confirmed by Frederickson et al. (1981). The observations led to numerous investigations of the significance of zinc in the most stained part of hippocampus, the mossy fiber system (Crawford et al. 1973; Danscher et al. 1975; Euler 1962; Fleischhauer and Ohnesorge 1958; Frederickson and Howell 1984; Frederickson et al. 1981; Hesse 1979; Howell et al. 1984; Otsuka and Ibata 1966; Szerdahelyi 1982).

When Timm's sulphide silver method was described in 1958 (Timm 1958), a new tool for localization of metals in the brain was introduced and the presence of metal in the hippocampal mossy fiber zone (McLardy 1960) and in the mossy fiber boutons (Haug 1967; Ibata and Otsuka 1969) was established. Recently the silver amplified metal sulphides have been localized in the synaptic vesicles not only in the mossy fiber boutons (Danscher 1984c), but in stained boutons throughout the telencephalic structures (Friedman and Price 1984; López Garcia et al. 1984; Pérez-Clausell and Danscher 1985). A major problem, however, is that Timm's method is believed to be rather unspecific because any metal that can be transformed to a metal sulphide could theoretically be silver amplified by physical development, that is, be visualized by Timm's method (Timm 1958).

In order to determine whether Timm's method demonstrates the same metal(s) as the intravital dithizone method animals were treated with dithizone prior to being perfused with sodium sulphide. No Timm staining could be found in the brains after this procedure, suggesting that dithizone prevented the formation of metal sulphides (Danscher 1984a, b; Haug and Danscher 1971; Otsuka and Ibata 1966). Experiments with other chelating agents supported this interpretation: diethyldithiocarbamate (Danscher et al. 1973), oxine and dimercaprol 1-10-phenantroline and 2,2-dipyridyl (Danscher and Fredens 1972; Schrøder et al. 1978) also block a subsequent Timm staining.

It is known that dithizone as well as the other chelating agents affected behavior (Aigner et al. 1967; Danscher 1976; Fleischhauer and Ohnesorge 1958; Maj and Vetulani 1970; Moore 1969) and synaptic transmission (Crawford et al. 1973; Danscher et al. 1975; Hesse 1979).

When the intravital selenium method was introduced (Danscher 1982) it was found that the general neuropil staining pattern not only was similar to the Timm pattern but also reacted in the same way to intravital treatment with chelating agents. If for example animals were treated with dithizone 15 min prior to a treatment with sodium selenide, no staining was found, suggesting that the two methods demonstrated the same metal(s).

In Stampfl's (1959) extensive work on the use of dithizone as an intravital marker for zinc in different organs other than the brain, he also examined the carbon tetrachloride extracts with a multielement detection method. Only zinc was found.

Preliminary results from the CNS indicated that the red color seen in the whole telencephalon structures after intravital dithizone treatment is due to zinc dithizonate (Danscher 1984b). In the present report, data from PIXE analysis of carbon tetrachloride extracts from brains and spinal cords intravitally exposed to dithizone suggest that

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the metal visualized in the neuropil with the three methods is zinc.

## Materials and methods

Ten Wistar rats with an average weight of 150 g were used in the study. Samples to be analyzed were prepared in the following way:

*1)* All the surfaces in contact with the samples were stainless steel, quartz, teflon and polyethylene. All the tools and containers were thoroughly cleaned: 24 h in dekonex, rinsed in twice deionized water, followed by another 24 h stay in warm (50° C) conc. nitric acid. After a rerinse in deionized water, the containers were dried in an oven (40° C).

*2)* The cleaned empty containers were labeled and weighed.

*3)* A fresh dithizone solution was prepared for each series of experiments: Sodium dithizone (-diphenylthiocarbazone) 125 mg was poured into 1.25 $cm^3$ absolute ethanol and 5 drops of concentrated $NH_4OH$ were added. When the dithizone was completely dissolved, 25 ml distilled water was added resulting in a 0.5% dithizone solution.

*4)* Rats were anesthetized with a 5% Nembutal solution (50 mg/kg), intraperitoneally injected with dithizone (150 mg/kg) and allowed to survive for 20 min. The animals were then decapitated, the brain and spinal cord were removed, rinsed in twice distilled water and dissected in a flow bench.

*5)* The dissected samples of brain and spinal cord were separately placed in the preweighed containers, frozen and freeze-dried.

*6)* Containers with the lyophilized samples were weighed and the tissue was homogenized in the containers using a stainless steel pestle. Twice distilled water was added in a ratio of 40 ml water per gram dry weight. After 60 min the samples were poured into a 25 ml separating funnel. The same ratio of carbon tetrachloride was added to the sample and the funnel was shaken for 5 min, allowed to stand for 10 min and then shaken for another 5 min. After 30 min the carbon tetrachloride extract from brain samples was collected and placed in a new container and was directly used for the PIXE study. Samples from spinal cords were collected after 60 min. The carbon tetrachloride was collected after 60 min because the two fractions were more difficult to separate.

*7)* 2 µl of the dithizonate containing carbon tetrachloride extracts were placed on a neutronpore filter and allowed to dry.

*8)* The samples, deposited on 1 mg/$cm^2$ Nucleopore filter material with 8 µm pore size (Nucleopore Corporation – Pleasanton, USA), were placed in a vacuum and irradiated by a beam of 2.55 MeV protons from a Van de Graaf particle accelerator. The beam current used in this case was 20 µA over a circular beam cross section of 6 mm diameter. Two rats not treated with dithizone served as controls.

Good beam homogeneity over this cross section was achieved by using a 0.6 mg/$cm^2$ gold foil as a beam diffuser and defining the beam size close to the target by a set of circular collimators. The X-rays resulting from the interaction between the protons and the sample atoms were detected by an energy dispersive Si (Li) X-ray detector with an active area of 80 $mm^2$ placed at an angle of 135° relative to the beam direction. The total charge collected in each measurement was 15 µC (microcoulomb). To decrease the number of low energy events registered in the X-ray detector, 155 µm of mylar was placed in front of the detector window. To diminish the effect of spurious peaks in the X-ray spectra, caused by pulse pile-up, a triggered beam pulsing system was employed (Koenig et al. 1977).

The ray events were registered by a minicomputer based data acquisition system.

## Results

Carbon tetrachloride extracts of dithizonate from brains and spinal cords were found to contain zinc (Fig. 1, $Zn^{(K_\alpha)}$ and $ZN^{(K_\beta)}$ and traces of copper (Fig. 1, $Cu^{(K_\beta)}$. Control

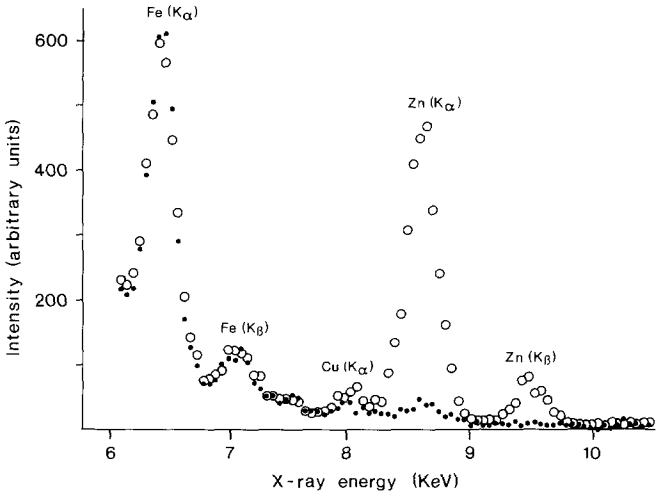


**Fig. 1.** Part of X-ray spectra recorded from carbon tetrachloride extracts from rat brain. • Spectra from animals not treated with dithizone. o Spectra from dithizone treated animals

solutions of sodium dithizonate (used for the intravital treatment) and carbon tetrachloride extracts from control animals not treated with dithizone had low levels of the two elements (Fig. 1). The iron peaks seen in both experimental and control samples are a result of scattered protons hitting the stainless steel chamber.

## Discussion

It has been suggested earlier that the three principal histochemical methods for metal detection, Timm's sulphide silver method, the selenium and the dithizone methods, demonstrate the same metal in the CNS, and that this metal might be zinc (Danscher 1984c). Preliminary studies had supported this suggestion (Danscher 1984b), and the present study reveals that an overwhelming part of the histochemical products are based on zinc. This metal is shown by the sulphide silver method to be located in synaptic vesicles of stained boutons (Danscher 1984a; Friedman and Price 1984; Pérez-Clausell and Danscher 1985). However, traces of copper were also found in the carbon tetrachloride extracts.

The elemental composition of the samples was analyzed using Particle-Induced X-ray Emission (PIXE) (Johansson and Johansson 1976). PIXE has during the past decade found increasing use in the analysis of biological and clinical samples (Kemp and Danscher 1979; Walter et al. 1974) since it is especially well suited for use with small samples as for example thin (10–60 µm) sections of tissue or dried liquid samples.

The traces of copper in the present material could be caused either by contamination or reflect the real presence of small amounts of chelatable copper in the brain. Timm (1961) demonstrated the presence of copper in liver cells by his Mg-dithizonate-silver method, and Szerdahelyi and Kása (1984) found copper in glial cells around the Purkinje cells using the same method.

If brain sections that have been soaked in an ethanol solution containing sulphide ions and then exposed to weak acids (which remove zinc sulphide, but leave copper sulphide accumulations intact), are exposed to physical devel-

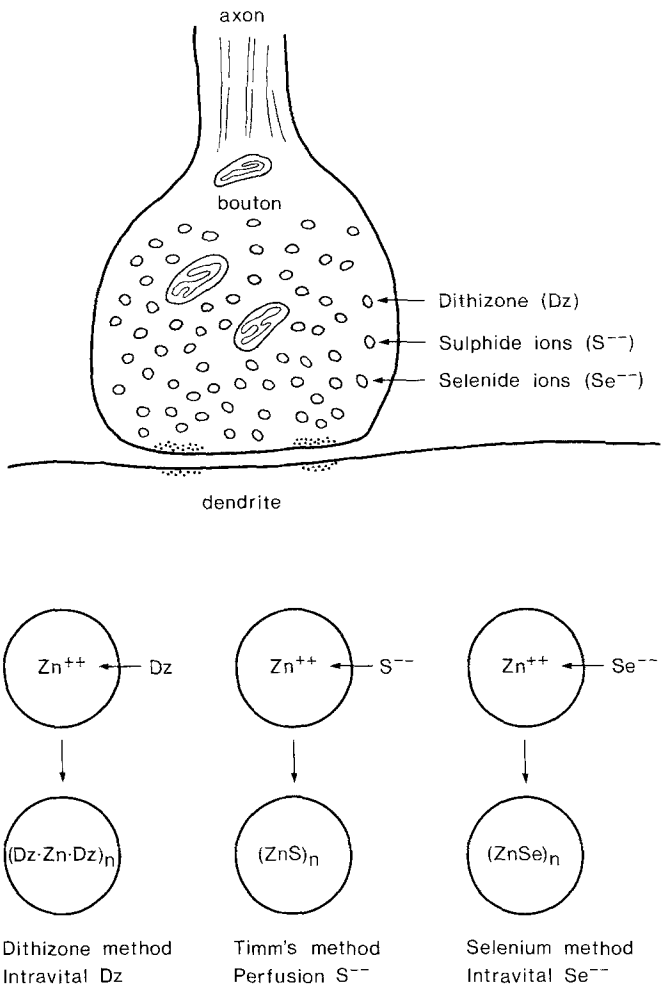


**Fig. 2. a)** Bouton containing vesicular zinc that can be bound to either dithizone (*Dz*), sulphide ions ($S^{--}$) or selenide ions ($Se^{--}$). **b)** After intravital treatment dithizone or selenium ions move into the synaptic vesicles and accumulate as zinc dithizonate, respectively zinc selenide. After perfusion with sulphide ions zinc sulphide precipitates in the vesicles. Zinc sulphide and zinc selenide accumulations can be silver amplified by physical development or autometallography

opment, precipitates are found in glial cells and in the somata of some of the neurons (Szerdahelyi and Kása 1984; Timm 1961).

Lenglet et al. (1984) by way of micro-PIXE found both copper and zinc in rat hippocampus. The micro-PIXE element distribution pattern for zinc was "comparable" to the Timm and selenium patterns, copper was found to be evenly distributed in the hippocampus.

The size of the chelatable zinc pool, DTS-zinc, compared to the non-chelatable is at present under investigation (Howell et al. 1985). It has been suggested that DTS-zinc in the mossy fiber region constitutes about 10% of the total hippocampal zinc (Frederickson et al. 1984). Zinc seems to be important for transmission in the mossy fiber-CA3 synapses (Crawford et al. 1973; Danscher et al. 1975; Euler 1962; Hesse 1979; Howell et al. 1984). Still it is surprising that after intravital treatment with the chelating agent diethyl-dithiocarbamate (Danscher et al. 1975) or sodium selenite (Danscher 1982), where seemingly all of the DTS-zinc is bound for tens of minutes, treated rats still function with some behavioral competence.

The dithizone, Timm's sulphide silver and the selenium methods are believed to be based on the following events: The anions (Dz, $S^{--}$, $Se^{--}$) move into the synaptic vesicles where free zinc ions, or zinc ions loosely bound to molecules, are precipitated and create discrete accumulations, possibly crystals of zinc dithizonate, zinc sulphide or zinc selenide (Fig. 2). The zinc dithizonates can be seen directly while ZnS and ZnSe have to be silver amplified – that is, subjected to physical development or autometallography. There is no doubt that each of the three methods is a valuable tool for further studies on the significance of DTS-zinc in synaptic transmission.

## References


Aigner A, Hornykiewicz O, Lisch H-J, Springer A (1967) Beeinflussung der Gehirn-Katecholamine, der Spontanaktivität und der L-DOPA Hyperaktivität durch Diäthyldithiocarbamat. Med Pharmacol Exp 17:576–585

Crawford IL, Doller HJ, Connor JD (1973) Diphenylthiocarbazone effects on evoked waves and zinc in the rat hippocampus. Pharmacologist 15:197

Danscher G (1976) Heavy metals in the hippocampal region. Some aspects of localization, function and content with special emphasis on the effect of chelating agents. Thesis, University of Aarhus, Denmark

Danscher G (1982) Exogenous selenium in the brain. A histochemical technique for light and electron microscopical localization of catalytic selenium bonds. Histochemistry 76:281–293

Danscher G (1984a) Similarities and differences in the localization of metals in rat brains after treatment with sodium sulphide and sodium selenide. In: Frederickson CJ, Howell GA, Kasarskis E (eds) Neurobiology of zinc, Part A. Alan R Liss, New York, pp 229–242

Danscher G (1984b) Do the Timm sulphide silver method and the selenium method demonstrate zinc in the brain? In: Frederickson CJ, Howell GA, Kasarskis E (eds) Neurobiology of zinc, Part A. Alan R Liss, New York, pp 273–287

Danscher G (1984c) Dynamic changes in the stainability of rat hippocampal mossy fiber boutons after local injection of sodium sulphide, sodium selenite, and sodium diethyldithiocarbamate. In: Frederickson CJ, Howell GA, Kasarskis E (eds) Neurobiology of zinc, Part B. Alan R Liss, New York, pp 177–191

Danscher G, Fredens K (1972) The effect of oxine and alloxan on the sulfide silver stainability of the rat brain. Histochemie 30:307–314

Danscher G, Haug F-MS, Fredens K (1973) Effect of diethyldithiocarbamate (DEDTC) on sulphide silver stained boutons. Reversible blocking of Timm's sulphide silver stain for "heavy" metals in DEDTC treated rats (light microscopy). Exp Brain Res 16:521–532

Danscher G, Shipley MT, Andersen P (1975) Persistent function of mossy fibre synapses after metal chelation with DEDTC (Antabuse). Brain Res 85:522–526

Euler C von (1962) On the significance of the high zinc content in the hippocampal formation. In: Passouant P (ed) Physiologie de l'hippocampe. Centre National de la Recherche Scientifique, Paris, pp 135–145

Fleischhauer K, Ohnesorge FK (1958) Zur Pharmakologie des Dithizon. Naunyn-Schmiedeberg's Arch Exp Pathol Pharmakol 235:63–77

Frederickson CJ, Howell GA (1984) Merits and demerits of zinc dithizonate histochemistry. In: Fredericksen CJ, Howell GA, Kasarskis E (eds) Neurobiology of zinc, Part A. Alan R Liss, New York, pp 289–303

Frederickson CJ, Howell GA, Frederickson MH (1981) Zinc dithizonate staining in the cat hippocampus: Relationship to the

mossy-fiber neuropil and postnatal development. Exp Neurol 73:812–823

Friedman B, Price JL (1984) Fiber systems in the olfactory bulb and cortex: a study in adult and developing rats, using the Timm method with the light and electron microscope. J Comp Neurol 223:88–109

Haug F-MS (1967) Electron microscopical localization of the zinc in hippocampal mossy fibre synapses by a modified sulphide silver procedure. Histochemie 8:355–368

Haug F-MS, Danscher G (1971) Effect of intravital dithizone treatment on the Timm sulfide silver pattern of rat brain. Histochemie 27:290–299

Hesse GW (1979) Chronic zinc deficiency alters neuronal function of hippocampal mossy fibers. Science 205:1005–1007

Howell GA, Welch MG, Frederickson CJ (1984) Stimulation-induced uptake and release of zinc in hippocampal slices. Nature 308:736–738

Howell GA, Pérez-Clausell J, Kemp K, Danscher G (1985) Chelatable zinc in the rat CNS. In preparation

Ibata Y, Otsuka N (1969) Electron microscopic demonstration of zinc in the hippocampal formation using Timm's sulphide silver technique. J Histochem Cytochem 17:171–175

Johansson SAE, Johansson TB (1976) Analytical application of particle induced X-ray emission. Nucl Instrum Methods 137:473–516

Kemp K, Danscher G (1979) Multi-element analysis of the rat hippocampus by proton-induced X-ray emission spectroscopy (phosphorus, sulphur, chlorine, potassium, calcium, iron, zinc, copper, lead, bromine, and rubidium). Histochemistry 59:167–176

Koenig W, Richter FW, Steiner U, Stock R, Thielman R, Wätjen U (1977) Trace element analysis by means of particle induced X-ray emission with triggered beam pulsing. Nucl Instrum Methods 142:225–229

Lenglet WJM, Bos AJJ, Stap CCAH, Vis RD, Delhez H, Hamer CJA (1984) Discrepancies between histological and physical methods for trace element mapping in the rat brain. Histochemistry 81:305–309

López Garcia C, Molowny A, Pérez-Clausell J (1984) Volumetric and densitometric study in the cerebral cortex and septum of a lizard (*Lacerta galloti*) using the Timm method. Neurosci Lett 40:13–18

Maj J, Vetulani J (1970) Some pharmacological properties of N,N-disubstituted dithiocarbamates and their effect on the brain catecholamine levels. Eur J Pharmacol 9:183–189

Maske H (1955) Über den topochemischen Nachweis von Zink im Ammonshorn verschiedener Säugetiere. Naturwissenschaften 42:424

McLardy T (1960) Neurosyncytial aspects of the hippocampal mossy fibre system. Confin Neurol (Basel) 20:1–17

Moore KE (1969) Effects of disulfiram and diethyldithiocarbamate on spontaneous locomotor activity and brain catecholamine levels in mice. Biochem Pharmacol 18:1627–1634

Otsuka N, Ibata Y (1966) Über die quantiativen Veränderungen des Zinkgehaltes in der Hippocampusformation nach der Dithizon-Zufuhr. Arch Histol Jpn 27:419–424

Pérez-Clausell J, Danscher G (1985) Intravesicular localization of zinc in rat telencephalic boutons. A histochemical study. Brain Res 337:91–98

Schrøder HD, Fjerdingstad E, Danscher G, Fjerdingstad EJ (1978) Heavy metals in the spinal cord of normal rats and of animals treated with chelating agents: a quantitative (zinc, copper, and lead) and histochemical study. Histochemistry 56:1–12

Stampfl VB (1959) Die intravitale histochemische Darstellung des Zinks durch Dithizon. Acta Hisotchem 8:406–447

Szerdahelyi P (1982) Lack of correlation between trace metal staining and trace metal content of the rat hippocampus following colchicine microinjection. Histochemistry 74:563

Szerdahelyi P, Kása P (1984) Histochemistry of zinc and copper. Int Rev Cytol 89:1–33

Timm F (1958) Zur Histochemie der Schwermetalle. Das Sulfid-Silber-Verfahren. Dtsch Z Gesamte Gerichtl Med 46:706–711

Timm F (1961) Der histochemische Nachweis des Kupfers im Gehirn. Histochemie 2:332–341

Walter RL, Willis RD, Gutknecht WF, Joyce JM (1974) Analysis of biological, clinical, and environmental samples using proton-induced X-ray emission. Anal Chem 46:843–855