

Invited Review

⁶⁵Zn in studies of the neurobiology of Zinc

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Summary. The presence of Zinc in the mossy fibre system of the Hippocampus is the most thoroughly studied of Zinc relation to the CNS, but many other areas of the CNS are Zinc-containing.

Many methods have been used in order to investigate the above mentioned relation, most of them being based on histochemistry and physical measurement. ⁶⁵Zn trace has also been used, but scarcely, probably due to the difficulty of radioisotope handling.

In the present review we focus on ⁶⁵Zn studies in the CNS and comment on their advantages and disadvantages.

Key words: ⁶⁵Zn, Zinc, CNS

Introduction

The discovery of the importance of the role that vitamins play in the functioning of the metabolic system in human beings by making themselves widely available to simpler chemical elements that in very small quantities are also indispensable for said functioning, focused biochemical research efforts from the end of the last century on the study of metals indispensable for health and even life. Because the quantities of these metals found in living organisms were so small they were called Trace Elements.

Very soon Raulin (1869) showed - in the Botanical field - the importance of Zinc in the growth and development of the *Aspergillus Niger* fungus. Henceforth, and during the first half of this century the participation of this heavy, bivalent metal was continuously demonstrated in numerous enzymatic activations throughout the entire biological scale, including Man. In the 1950s the development of histochemical methods also allowed research into this heavy metal to be carried out in the morphological field, and the intravital Dithizone methods or those heavy metal detection methods using Silver Sulphur allowed Maske (1955),

Fleischhauer and Hortsman (1957), Timm (1958) and Mc. Lardy (1960) to demonstrate, without a shadow of doubt, the presence of Zinc in the brain of mammals.

At the same time the methodology of the study of the presence of Zinc in human beings by the detection of its ⁶⁵Zn radioisotope was also developed. The techniques based on the detection of the radioisotope consisted in, whatever the case, a bridge union between the biochemical and morphological studies, since the tracer seemed to behave, except on some occasions, reasonably well to extrapolate the observations of the behaviour of stable Zinc. An example of this is shown in the early works of Montgomery et al. (1943) and Sheline et al. (1943) based on the measurement of radioactivity in the organic samples, or the first autoradiographs of this radioisotope in the pancreas (McIsaack, 1955).

In the context of general studies of this radioisotope the first data are found that relate ⁶⁵Zn to the Central Nervous System in works by Durbin et al. (1957) and Millar et al. (1961). But the work of V. Euler (1962) is possibly the key work that centres the histochemical results of the localizations of stable Zinc and the detection of ⁶⁵Zn in the Nervous System. In the above, the histochemical electrophysiological and autoradiographic results in the rat hippocampus are compared and as a consequence the presence and importance of Zinc in the function of this nervous territory were later confirmed by Crawford and Connor (1972). The work of Hassler and Soremark (1968) also merits mentioning. In their work a thorough autoradiographic study at macro-autoradiographic level demonstrated the uptake of ⁶⁵Zn in various parts of the CNS, especially in the hippocampus, brain cortex, cerebellum, etc. This was later collaborated by Akai et al. (1979) and by Dencker and Tjälve (1979) using more modern autoradiographic procedures for the whole body. However, all these studies were carried out in low resolution, which will be discussed in the technical section. Anyway, Frederickson (1989) in his review «The Neurobiology of Zinc and Zinc containing neurons» points out the technical difficulties of the radioisotope and takes importance away from the possibilities of the autoradiographic technique in this particular case. This point of view is also debateable and thus will be discussed in the

corresponding section.

The other procedure for the detection of ⁶⁵Zn in the CNS was its external detection by all the possible methods applicable to gamma transmitters. The early works of Montgomery et al. published in 1943 mark the first approach to the radiotracer of ⁶⁵Zn in dogs and rats. The approach to turnover in the CNS comes later and is owed to Davson and Segal (1970), and Czerniak and Haim (1971). However, it was Kasarskis (1984a,b) who totally centred the study, concluding that ⁶⁵Zn penetrates the CNS in places still not anatomically defined, being uptaken by neurons and glia, and transported distally via a slow axonal movement as postulated by Knull and Wells (1975), even speculating its relation with tubuline. In these conditions the radioisotope would suffer a relevantly uniform distribution in the brain. The excess of ⁶⁵Zn seems to be eliminated by the CSF and returns to the plasma through the choroid plexus, without excluding the possible return via the intracerebral capillary wall. Frederickson (1989), making use of the work of Sato et al. (1984), centres the biological half-life of the CNS in 22 days, the turnover for ⁶⁵Zn associated with myelinic fraction being faster than that associated with synaptosomes. Dieniel and Pulsinelli (1986) did not find any significant modifications in the localization of ⁶⁵Zn in ischemia-damaged brain. A new effort to explain the turnover of ⁶⁵Zn in the brain was offered by Franklin et al. (1992), work in which after 30 minutes of circulation in the blood there is an uptake of ⁶⁵Zn from the blood to the brain in one direction and in a constant inflow of approximately 5×10^{-4} ml/min/g. In times inferior to 30 minutes the flow is two-directional and greater than the above mentioned. As a conclusion they indicate the possible existence of a cerebral compartment of fast change which is not the CSF. However, the same authors find that the permeability of ⁶⁵Zn in the choroid plexus is 12 times greater than in cerebral capillaries, despite the fact that the inflow of Zn via this path is only 5% of that flowing through said capillaries. Having verified the dynamic study using autoradiography the existence of regional variations was demonstrated, the lowest levels being in white substance and the highest in the hippocampus.

The turnover of ⁶⁵Zn in the CNS

All the existing data (Knull and Wells, 1975; Kasarskis, 1984a,b; Sato et al., 1984; Pullen et al., 1990, 1991) imply a rapid incorporation of the radioisotope in the CNS when it has been administrated through the bloodstream. But it also stands out in quantities much smaller than could be expected in injected doses and the place and type of incorporation is debated. However, a series of aspects concerning the above start to be evident:

- The places bereft of encephalo-CSF barrier (postrema area, basal area of the hypothalamus) offer no obstacle to the diffusion of the radioisotope from the CSF to the CNS (Kasarski, 1984b).

- The choroid plexus accumulates ⁶⁵Zn and maintains an enormous difference in concentration with respect to the CSF, in which 24 hours after injection into the bloodstream, activity is practically non-existent according to the majority of authors.

- In the CNS the radioisotope is taken up by some glial cells and by neurons, where it is transported via a slow axonal flow (Knull and Wells, 1975).

- The excess ⁶⁵Zn in the CNS is eliminated rapidly although it is still not clear whether this is carried out through the CSF or directly through the brain capillaries (Frederickson, 1989).

- Although the incorporation of the radioisotope in the CNS is very fast its capillary permeability is low, 12 times less than that of the choroid plexus (Franklin et al., 1992).

In conclusion, ⁶⁵Zn coming from the blood is taken up quickly by the CNS, but as Kasarskis (1984b) and Frederickson (1989) asserted, we are very far from precisely defining the anatomical mechanism and place where this incorporation is produced. Therefore, there are two possible although debateable hypotheses:

A. ⁶⁵Zn is filtered by an active transport and controlled from the choroid capillaries to the CSF and from there passes very rapidly to the CNS. Only using territories without CSF/CNS barriers? Using the CSF/CNS barrier as well, with a specific facilitating mechanism?

B. The radioisotope is filtered directly through the brain capillaries to the nervous tissue by a slow but selective and efficient process.

Both hypotheses are compatible, and both seem to be supported by present-day bibliography.

From here the radioisotope is incorporated into the nervous tissue in an apparently quite general manner, although in an inferior quantity in white substance with respect to grey substance and with evident differences of regional concentration with respect to the hippocampus which manifests itself to be more receptive according to studies by Sato et al. (1984) - as much as 2.5 times greater than in the cerebellum. However, in the same work, the uptake peaks of the cellular fractioning over a period of 10 weeks show quite similar profiles. This data slightly differs from that of Dieniel and Pulsinelli (1986) but this can be justified by the different methodology used. Here we show uptake graphs that we have made from the date from these authors, and we include our own study of daily turnover in the first two weeks (Pérez-Castejón et al., 1990; Vera-Gil et al., 1990) - Graphs I-V.

As a whole it does not seem that great differences exist between the hippocampus, the striate, the cerebellum and the cortex (Graphs I, V) so it has to be though that perhaps the apparent difference in uptake is due to the structural concentration, confined in the hippocampus to a very small anatomical territory. However, by comparison, the uptake peaks between the most receptive territory - the hippocampus - and the least receptive - the cerebellum - present, in principle,

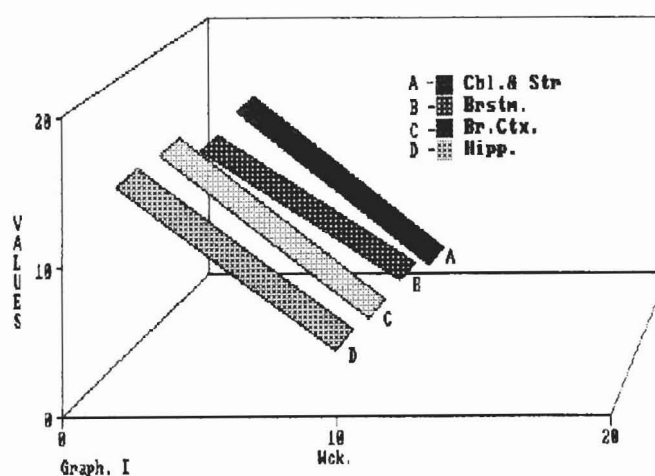
significant values (Graph IV). In the graphs showing uptake by tissue fractions (Graphs II, III) a similarity of profile is appreciated between the cellular components and the very significant difference in the ^{65}Zn fraction in the cytosol, which may imply appreciable functional differences.

On the other hand, the low proportion of differential uptake between Nervous territory and the rest of the body is evident. Here the radioactivity counts described by all the authors amounts to a ratio of 0.1.

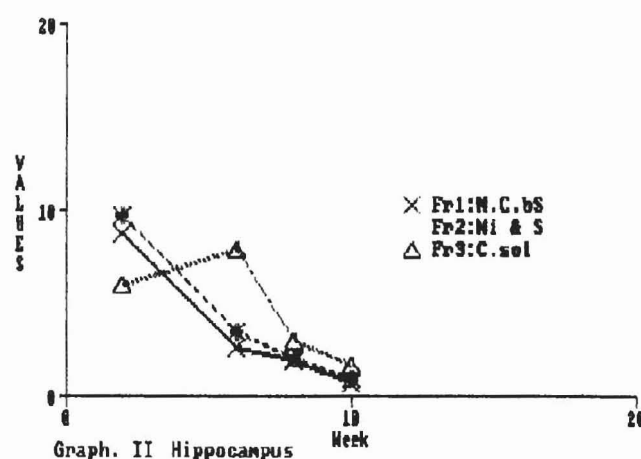
Nevertheless, summarizing, the ^{65}Zn incorporated into the CNS persists for a long period of time, with some differences according to different bibliographies. We can estimate that, in general, its biological half-life in this territory is produced approximately in the fourth

or fifth week, which in our opinion confirms all the functional hypotheses put forward for stable Zinc by Frederickson (1989). However, at present, we do not believe that the data allows us to specifically distinguish which of them is particular.

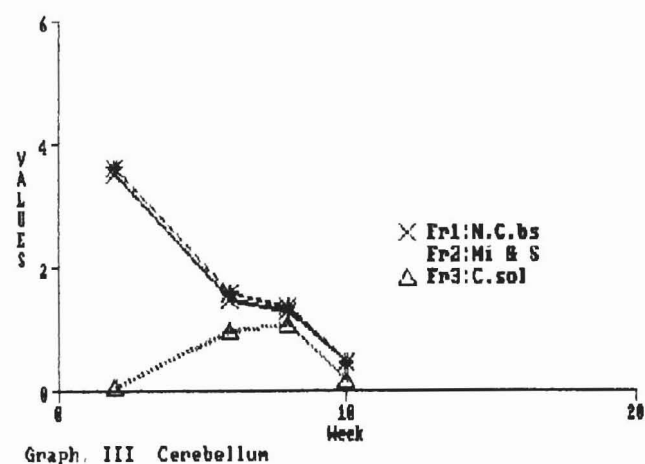
The retention as well as the flow of ^{65}Zn in the CNS appear justified in the bibliography and, once more, the capillaries seem to be the place where the extrusion of the radioisotope towards the blood - in a selective manner - takes place, sharing the main path CSF/choroid plexus as described by Karsarskis (1984b). The peaks and valleys of uptake observed in brain dynamics during the first two weeks (Graph V) undoubtedly obey the functioning requirements of the CNS while the radioisotope is very available.



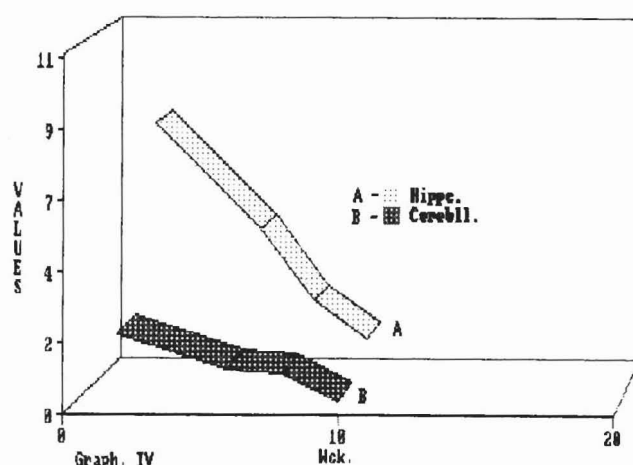
Graph I. A: cerebellum and Striate; B: brainstem; C: cerebral cortex; D: hippocampus; X: chronology of sample in weeks; V: values of ^{65}Zn expressed in percentage of the total doses administered measured in the samples. Graph made with data by Dienel and Pulsinelli (1986).



Graph II. Hippocampus. Fr1 is the nuclei fraction, whole cells and large synaptosomes. Fr2 is the mitochondria and synaptosome fraction. Fr3 is the cytosol fraction. x: chronology of the samples in weeks. y: values of ^{65}Zn in counts per minute (CPM). Graph made with data by Sato et al. (1984).



Graph III. Cerebellum. Fr1 is the nuclei fraction, whole cells and large synaptosomes. Fr2 is the mitochondria and synaptosome fraction. Fr3 is the cytosol fraction. x: chronology of the samples in weeks. y: values of ^{65}Zn in CPM. Graph made with data by Sato et al. (1984).



Graph IV. Comparison of the average values in the hippocampus (A) and cerebellum (B). The ordinate axes express the parameters as in Graphs II and III. Graph made with data by Sato et al. (1984).

Autoradiography with ^{65}Zn in the CNS

Autoradiographic results are mainly centred on the macroradiographic images shown by V. Euler (1961) for the hippocampus; by Hassler and Soremark (1968) for the rest of the CNS; and also the whole-body technique as in the works by Dencker and Tjälve (1979).

Contrary to what is generally thought the histoautoradiographic technique can be realized and, in fact, we have personally carried it out with ^{65}Zn , as did Franklin et al. (1992).

Frederickson (1989) adduced that «Unfortunately, the high gamma emissions (and resulting Compton electro scattering of ^{65}Zn) preclude cellular localization of the isotope». However, Rogers (1973) had already affirmed in his manual, «Techniques of Autoradiography», that gamma radiation «only betrays its presences through infrequent secondary electrons that can produce background», although he added that, «it only happens when the unloaded particle loses energy because of direct collision with electrons or nuclei. Given that such collisions are relatively infrequent, the particles can travel a considerable distance without exposing latent images». As a consequence he suggests that when one works with material susceptible to these kind of emissions the use of amplifiers (lead covering, etc.) is avoided and, as always the fading and background should be rigorously controlled. Because of this and because of how we learnt the technique from him and other colleagues in the English Autoradiography Club (Appleton, Williams, Blacket and Parry) in the 1970s, we are always very careful to balance the resolution factors playing with the sensitivity of the emulsion, the thickness of the layer, the size of its crystals and the double control of background, counting in the field the number of impacts inside and outside the tissue, within

the same preparation and fixing the hypothetical relation true/false impact in order to evaluate the reality of what we see in a more objective manner.

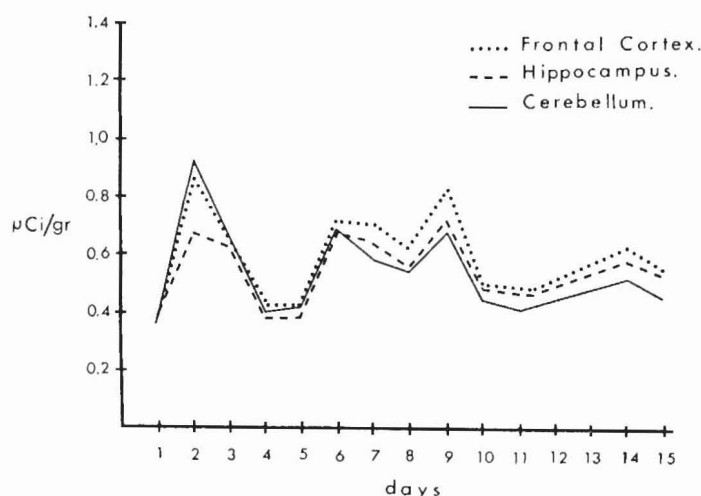
Having said this, in our opinion, conventional level histoautoradiography and optic microscopy are not only applicable but useful and their resolution very acceptable (Vera-Gil, 1974) as concerns the type of cells that display uptake. One thing that is certainly sure is that, in the case of ^{65}Zn , the concentration of impacts at this microscopic level is desperately poor in comparison with the brilliant histochemical images, and one has to have a great deal of experience, a great deal of technical knowledge, a lot of background control and one has to be very objective to evaluate the images. On the other hand, since the brain distribution of the radioisotope is dictated by its turnover, and the specificity of tissue uptake in each territory does by no means offer that of histochemical techniques used at EM level, things are even more difficult if we consider that even for the distribution of ^3H a previous fixation of Hotline and complicated statistical analyses are needed. As far as we know no ^{65}Zn Hotline has been achieved and our present-day, limited technical means have not allowed us to do it either. So our experience in ^{65}Zn at EM level has been developed in territories outside the CNS where there is a high concentration of radioisotope and only when the evidence of other techniques validated our results (Vera-Gil et al., 1981).

As proof of all that has been said, and apart from our own personal experiences, we now refer to the latest publication by Franklin et al. (1992) where an autoradiography with ^{65}Zn of the brain, carried out using conventional histological methods presented at very low microscopical magnification, proves to be very demonstrative of the concept in fashion.

Nowadays, what the autoradiographical technique tells us is that using conventional histological levels with great technical precaution we can extend our study of the localization of ^{65}Zn even to cellular groups with a uniform marking in all its components, which is quite sufficient if we take into account the excessive focus that has been centred on the hippocampus. Other nervous structures have shown the uptake of ^{65}Zn and histochemical positivity, the turnover studies demonstrating that they are not mere illusions, and to cite specific examples we can mention the hippocampus and the cerebellum (Figs. 1, 2).

With respect to the questions «Which conventional histochemical technique is the most appropriate for autoradiography with ^{65}Zn , and what are the ideal conditions?» the answer must be based on the following facts:

- All the conventional tissue processing techniques and the soluble substance techniques have been used. The results are markedly similar, which does not seem a determining factor, from which it can be deduced that the localization of the radioisotope is structurally stable. Nevertheless, some authors tend to lean towards the soluble substance technology, probably in order to avoid



Graph V. Comparative profile of ^{65}Zn uptake in CNS made from the average result taken from 6 samples from four animals sacrificed each day between the first and fifteenth day after intraperitoneal injection of 355 μCi of ^{65}Zn , measured in pit counter and converted into μCi per gram of tissue.

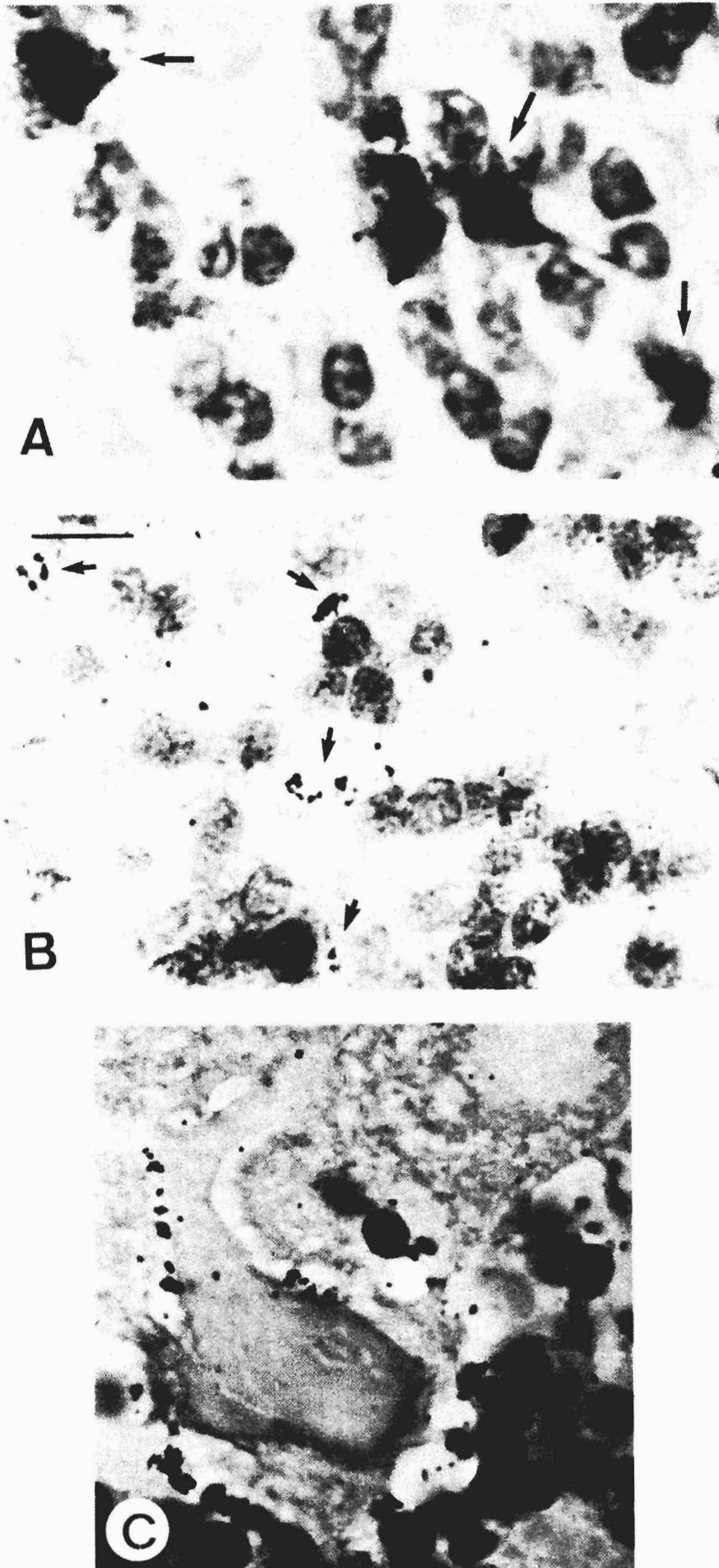


Fig. 1. ^{65}Zn in rat cerebellum. **A.** Control, Timm's technique showing dense positivity in compatible zones with glomerules in the cerebellar cortex layer of grains. **B.** Autoradiography with ^{65}Zn showing uptake in zones coincidental with 1A. **C.** Autoradiography with ^{65}Zn . Details of uptake in the periphery of a Purkinje cell. Scale Bar = 10 μm .

criticism (presumably unfounded in view of existing bibliography) or in order to try and find differences in the nature of the localization (an interesting measure of functional approach).

- As concerns the conditions for carrying out autoradiographic experiments, the one-injection system is the most efficient method of emulating the natural behaviour that Zinc would follow. In fact, the intraperitoneal injection of ⁶⁵ZnCl₂ carried in sterile saline serum is the best possible imitation except for oral administration, even though the latter has the disadvantage of the inevitable uncontrollability of the doses introduced given the peculiarities in intestinal absorption of Zinc. The direct intravascular injection is very safe with respect to the organ which is the object of study, but the possible territorial overload and consequent alteration in normality that this involves has to be evaluated since we are trying to follow a marker which will give us the key to what happens in normal conditions. The date of the sacrifice of the animal will be determined by the type of injection and the factors of concentration and metabolism that we can deduce from turnover studies. With the one-intraperitoneal injection our criteria was, in principle, to sacrifice on the sixth day following the general biological half-life described by Khristov (1965) for rats. In later, general turnover studies we took advantage of the opportunity to carry out autoradiographs on rats sacrificed daily, ranging the autoradiographs between the first and the fifteenth day

after sacrifice. Our impression from this, also corroborated by bibliography on turnover, is that each organ has its own radioisotope turnover. Therefore, it is impossible to generalize and, moreover, the differences from the moment that the uptake phase by an organ has been completed until the phase of depletion or consumption are significant as far as localization is concerned and of course very difficult to determine functionally using autoradiography. In this case it is evident that turnover studies are the most appropriate.

With regard to the sacrifice method in autoradiography, fast tissue fixation is the golden rule, so energetic and fast fixers acting, practically, on live tissue are advisable, as are the methods using freezing in liquid Nitrogen, with very rapid extractions of the sample. In any case one has to take into account that in this type of autoradiogram the essential thing is to be able to localize the impacts in the emulsion, the presence of tissue retraction or the attractive staining technique which is sometimes counterproductive if it interferes with the black of the emulsion grains exposed being of secondary importance.

The conditions of exposure, development and fixation in the autoradiographic procedure must be selected in accordance with the technique and emulsion employed. At present the direct application of X-ray plates seems somewhat archaic, but they have been used, above all in the early days. Stripping films are more appropriate when seeking whole body autoradiograms or not very

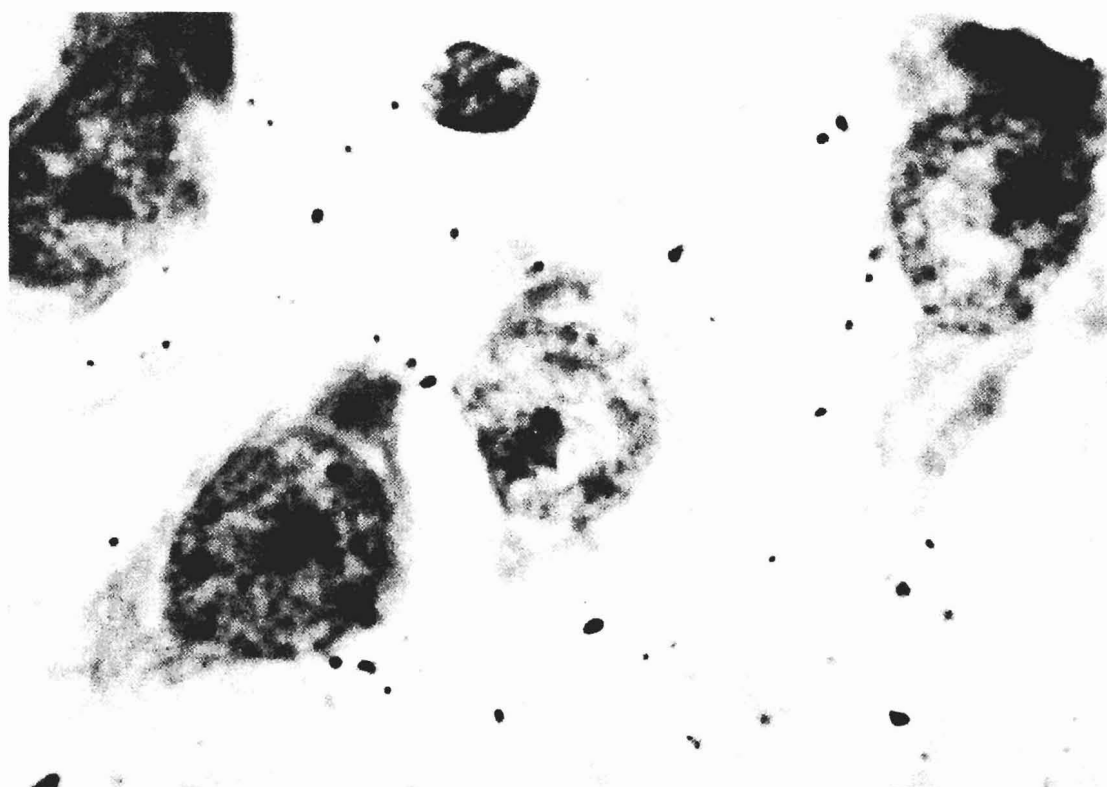


Fig. 2. Autoradiography with ⁶⁵Zn in rat hippocampus. Details of uptake in the vicinity of the H₃ pyramids, localization compatible with mossy endings. Scale bar = 10 μm.

high resolution autoradiograms. Unfortunately, the Kodak-AR-10, excellent for those purposes, is no longer produced so one has to resort to specifically ordered preparations (Franklin et al., 1992). In conventional histology and Dipping procedures, the Ilford L4 K5 or Kodak NTE emulsions are easily obtained and highly efficient.

For ^{65}Zn and conventional level histology our recommendation is: Ilford K5 emulsion 25-40%, and 4-6 weeks of exposure; Kodak D19 developer and Hypam fixer, with staining contrast after the autoradiographic procedure. In general, this system has given us good results even in the CNS.

Conclusions

The approach to studying the localization and significance of Zinc in the CNS does not often use the radiotracer with ^{65}Zn . However, when this method has been used it has proved its reliability and capacity to furnish unquestionable dynamic and morphological data.

The reasons generally offered to explain the limited use of this technique are:

1. With regard to turnover studies: in spite of the relatively low incorporation of the radioisotope in the CNS, it is still one of the most used techniques and has given more than enough samples of its capacity to provide data that histochemistry sometimes cannot, e.g. that the presence of Zinc in the CNS is much more common than exclusively histochemical works used to describe.

2. With regard to autoradiographic localization studies: apart from the reason mentioned earlier, two more must be added. Firstly, respectful terror of the physical characteristics of ^{65}Zn ; and secondly, which almost no one mentions but which is possibly more important, ^{65}Zn does not behave *in vitro* exactly as does stable Zinc since it does not displace itself to its last molecular binding when it has played its covalency capacity role creating a firm link (Davson and Eggleton, 1968). With reference to the first objection, throughout the text we have sufficiently discussed it and we seen that the result in the bibliography has been that those who have used the autoradiography technique in general have done so timidly and in very low resolution. Nevertheless, it should be added that in general those works belong to times in which technique was very limited and modern routines were not widely available. Today these have been modified and offer great capacity. On the other hand, no autoradiographic expert, given the physical conditions of the radiotracer and the expected low concentration, is going to dare increase microscope modification for fear of criticism from «referees» undoubtedly influenced by the opinions of very well-known authors who have never moved away from histochemical techniques. However, the solution is obvious: (a) analyze autoradiographic and histochemical images side by side. The most normal result is to observe that they validate each other; (b) make use of the

rich existing bibliography on the use of histochemical techniques. This is not our secret, it was foreseen and carried out earlier by V. Euler (1962) and we have attempted to follow his example in our approach. Working thus one realizes that the brilliant histochemical images sometimes produce the illusion of exclusively focusing attention on the most exuberant territories. The simplicity of autoradiography provides us with the best study, since it is possible to see the territory better without the histochemical jungle and thus localize and guarantee that it is Zinc that one sees. Unfortunately, histochemical techniques alone cannot avoid this doubt (although minimal on occasions) about which metal of similar chemical characteristics has been able to «falsify» the reaction expected.

When histochemistry and autoradiography coincide, not to accept what they demonstrate is merely a desire to ignore evidence. This sometimes happens.

Referring to the difference in behaviour between the radioisotope and its stable homonym is not as such an objection, but a fact to be taken into account when interpreting the functions of Zn from the autoradiograms or the dynamic trace. In the worst case it rules out the functions bound to molecules with strong links, as in the case of Carbonic Anhydrase (which is almost an advantage given the commonness of this enzyme). On the other hand, if one is cautious and waits patiently for the metabolic normalization of the tracer, giving it time to incorporate «*ex novo*» into the molecules needing Zinc, the problem is removed. In fact, dynamic studies carried out on animals subjected to a deficiency of Zinc have demonstrated the «avidity and seizure capacity» over ^{65}Zn after being administered (Bergman and Wing, 1974).

Finally, as a summary, we recommend associating the trace technique and the detection of ^{65}Zn technique to any study that involves the demonstration of the morphological and functional role of Zinc in Neurobiology.

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