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# Zinc uptake by brain cells: 'surface' versus 'bulk'

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**Abstract.** The uptake of zinc by cerebellar rat cultures upon exposure to $ZnCl_2$ solutions was comparatively investigated using two well known condensed matter physics techniques: synchrotron photoelectron spectromicroscopy and inductively coupled plasma atomic emission spectroscopy. The objective was to apply a strategy—well known in surface physics—to distinguish between 'surface' and 'bulk' phenomena. The results clearly demonstrate that exposure significantly enhances the bulk (cell cytoplasm) Zn concentration with respect to the physiological level, whereas the effect on the surface (cell membrane) is negligible.

## 1. Introduction

We investigated the zinc concentration in different types of rat cerebellar primary cultures, part of which had been exposed to zinc-containing solutions. The purpose was to study the exposure-stimulated zinc uptake, specifically the relative role of the cell's membrane (the 'surface') vs the cytoplasm (the 'bulk'). We found that the exposure does not significantly modify the membrane Zn concentration with respect to the physiological levels, whereas it does increase the zinc concentration in the cytoplasm.

These experiments are one example of growing applications of surface physics techniques in biophysics [1–6]. We used a standard approach [7] of surface science to distinguish between surface and bulk phenomena, by comparing results of experimental techniques with or without surface sensitivity. The surface-sensitive technique was the XSEM (x-ray secondary electron-emission microscopy) mode of synchrotron spectromicroscopy [8] and the bulk-sensitive technique was ICP-AES (inductively coupled plasma atomic emission spectroscopy) [9–11].

The results were quite straightforward: the bulk ICP-AES data revealed a clear increase in zinc concentration after exposure to the Zn solution. On the contrary, a very detailed series of spectromicroscopy studies did not reveal any significant increase, the detected zinc being at the same level as that found for non-exposed cultures. The same result was obtained for all varieties of brain cells included in the experiments: glial cells, granule and Purkinje neurons and astrocytes [12, 13].

The description of the study is organized as follows. Section 2 explains the experimental procedures; section 3 describes the data; section 4 discusses the two sets of data, comparing them to each other and drawing general conclusions.

## 2. Experimental techniques

The study was based on the comparison of the results of two different experimental techniques: synchrotron photoelectron spectromicroscopy [1–3, 5, 8] and ICP-AES [9–11]. Photoelectron spectromicroscopy is a recently developed technique described, for example, in references [8] and [14]. In a conventional photoemission experiment [7, 14] photoelectrons emitted by a macroscopic portion of a specimen are analysed to obtain information on a variety of its properties. The non-microscopic nature of these experiments is not the experimentalist's choice, but a consequence of the low signal level in a typical photoemission experiment.

Recent instrumentation advances [8] have made it possible to overcome this obstacle and to perform photoemission experiments in a submicron range of lateral resolution. The present experiments were conducted with

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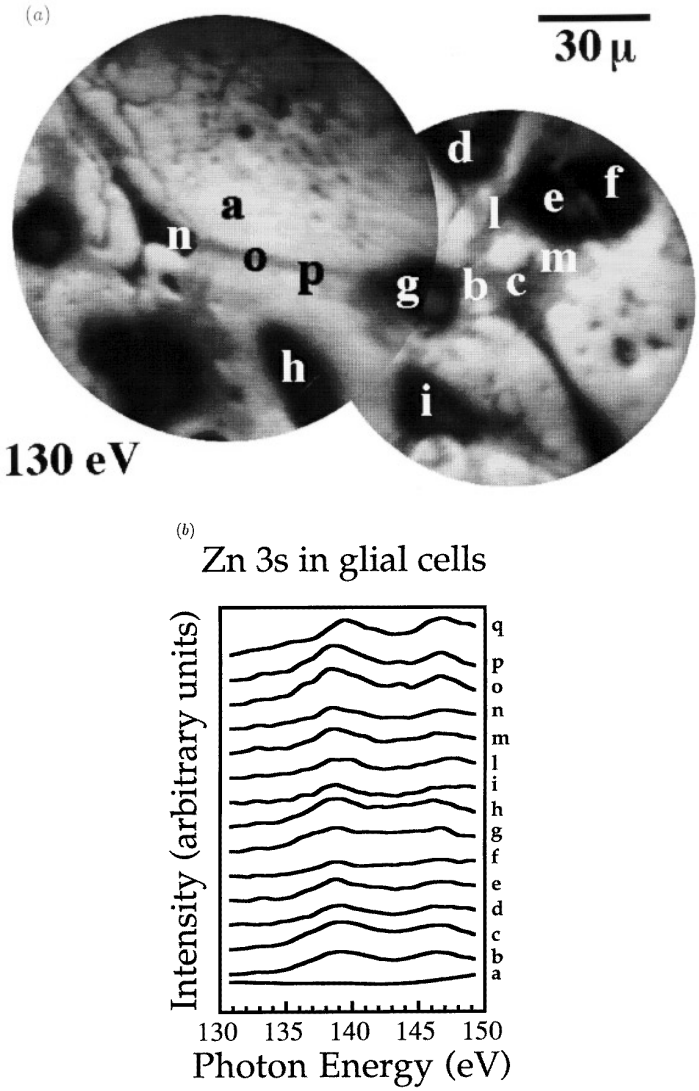


**Figure 1.** (*a*) Combination of two x-ray secondary electron-emission microscopy (XSEM) images of a portion of a culture with prevailing population of glial (type I) cells, after exposure to a 5 mM $ZnCl_2$ solution for 20 min. The photon energy was 130 eV. The letters identify the microscopic ($\approx 3 \times 3\mu m^2$) regions in which the spectra of figure 1(*b*) were taken. (*b*) XSEM (x-ray absorption) spectra from these regions. The data were background corrected, normalized and twice-smoothed over five-point sets. Curve a refers to a substrate area and is the only one not showing the typical [16] Zn3s absorption threshold lineshape. Curve q is a reference spectrum from a dried droplet of a zinc sulphate solution.

the XSEM (x-ray secondary electron-emission microscopy) variety of photoelectron spectromicroscopy [8].

Monochromatized x-ray photons were emitted by a synchrotron source, the 6 m toroidal grating monochromator (TGM) beamline of the Aladdin ring at the Wisconsin Synchrotron Radiation Center. The photons stimulated the emission of secondary photoelectrons, through intermediate energy-loss steps.

Gudat and Kunz demonstrated [15] that intensity–photon energy spectra taken in this way correspond to the x-ray optical absorption coefficient, detected with intermediate surface sensitivity (because of the low energy of the secondary photoelectrons). In the case of the XSEM, an electron-optics system is used to process the secondary photoelectrons, to image the geometric features of the specimen and to take spectra on microscopic areas.

Parallel tests were systematically performed to assess the possible presence of problems such as radiation damage or charging, with negative results. We are therefore confident that our present results are immune from such problems. Specifically, samples exposed for hours to the monochromatized x-ray beam (whose size was of the order of $0.3 \times 0.3$ $mm^2$) did not exhibit any detectable evidence of damage—spectral changes, for example. Substantial damage was instead observed after several minutes of exposure to a much more intense unmonochromatized beam (which was never used in our present experiments).

It should be noted that this kind of spectromicroscopy can investigate biological specimens without previous coating and labelling, and therefore in a state more similar to the natural one than in most electron microscopies. However, this approach is not feasible for the use of live specimens because of the necessity of a ultrahigh vacuum and therefore of previous dehydration of the sample. The

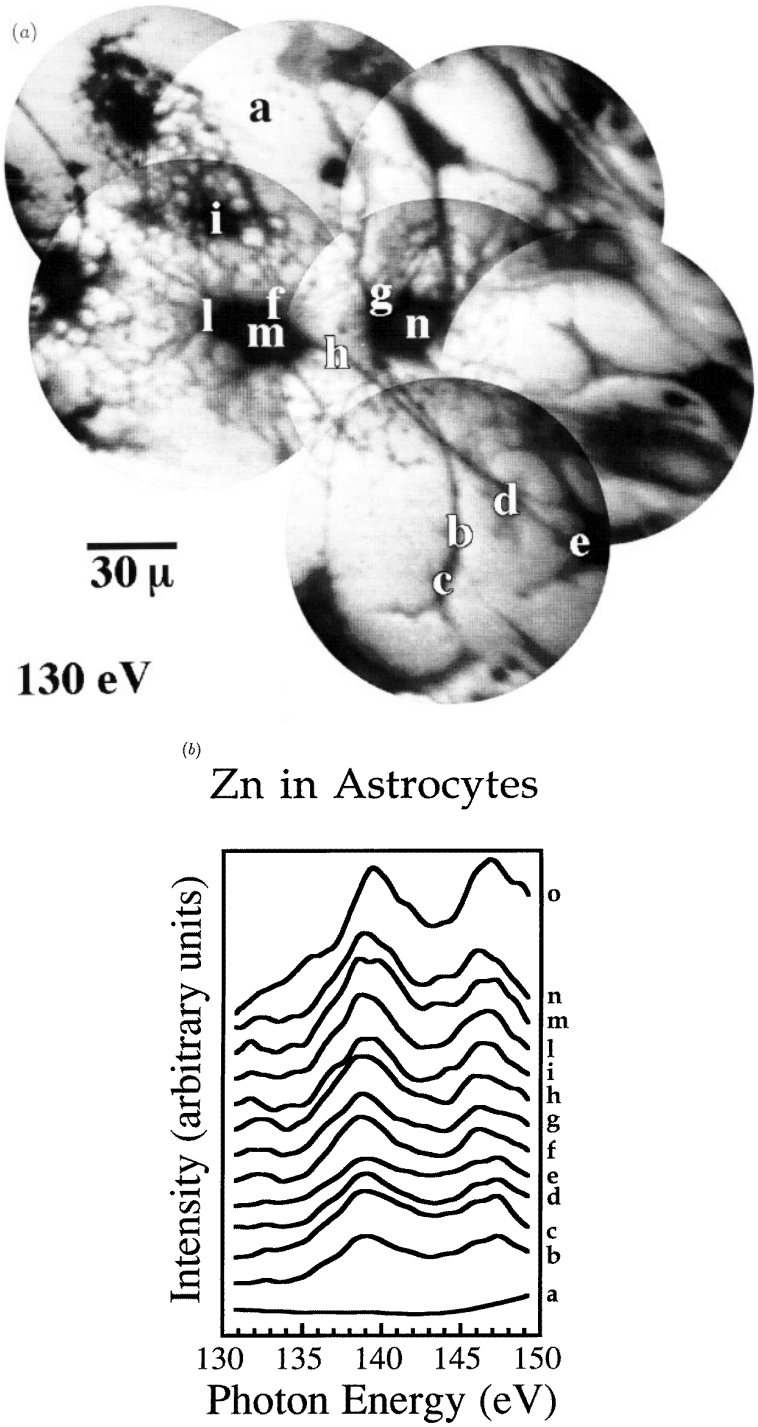


**Figure 2.** Data similar to those of figure 1, taken on a group of astrocytes (glial type II cells) found as a subpopulation of a culture similar to that of figure 1, with the same Zn exposure. Curves a and o are the substrate and reference spectra respectively.

XSEM technique has a chemical sensitivity limit of the order of 100 ppm [2].

The second of our experimental techniques, ICP-AES, performs chemical analysis on liquid samples. The liquid solution is heated to a cold-plasma temperature (8000 °C) by an electromagnetic field. At this temperature each element emits a specific near-ultraviolet light wavelength that can be easily identified.

Measuring the emission intensity at each wavelength, and comparing it to a standard reference, makes it possible to determine the concentration of each element in the solution. The sensitivity reaches 1 ppb. The conceptual background of the ICP-AES technique can be found, for example, in references [9–11].

We applied our experimental approach to a variety of primary cerebellar cell cultures. A detailed description

of our specimen preparation process can be found in the literature [1–6, 11, 12]. In short, cells extracted from rat cerebellum were allowed to grow for 7 or 8 days on gold substrates (for spectromicroscopy) or in 12-well Costar trays (for ICP-AES), pre-treated with 5 $\mu$g ml$^{-1}$ of poly-L-lisine solution. Selective techniques [11, 12] were used to obtain cultures with prevailing populations of different types of cerebellar cells: granule cell neurons, glial cells or Purkinje neurons. We also investigated astrocytes, found as a subpopulation in polygonal glial cell cultures.

At the end of the growth period, the cultures were washed and then they were either directly investigated or exposed, before investigation, to a $ZnCl_2$ solution in uptake buffer. Different exposure times (0–20 min) and different concentrations (1 or 5 mM) were used in the latter case. The exposure process included in some cases the use of excitatory amino acids in neurotoxic concentration (100 $\mu$M), either kainate or glutamate. Furthermore, some exposures were performed in the presence of an $Na_2S$ solution to induce the precipitation of zinc in the sulphide form.

After carefully washing to remove all the non-uptaken zinc, the cultures to be studied by spectromicroscopy were fixed with para-formaldehyde and dehydrated. Only part of the cultures selected for ICP-AES analysis were fixed; all of the ICP-AES cultures were suspended in $HNO_3$ 1 N to solubilize metal ions.

## 3. Results

Figures 1 to 7 show different sets of spectromicroscopy results; the ICP-AES findings are summarized in table 1. Specifically, each set of figures shows a spectromicroscopy image for a given type of cell, together with a series of XSEM spectra taken at different points of the same image.

Figure 1(*a*) refers to polygonal (type I) glial cells from a culture with prevailing population of cells of this kind. Figure 1(*b*) shows XSEM spectra taken in the correspondingly labelled microscopic areas of figure 1(*a*). The spectral range in each case includes the Zn3s x-ray absorption edge region and, in fact, the signal from that edge is clearly visible in all of the spectra. For comparison, the curve labelled a was taken on the substrate and shows no evidence of zinc. For reference, curve q was taken instead on a dried droplet of zinc sulphate solution in water.

Figure 2 refers to a group of astrocytes (glia type II) from the same culture of figure 1. In the spectra of figure 2(*b*), curves a and o are the substrate and the reference data. Figure 3 shows data of Purkinje neurons from a culture with prevailing cell population of this type. The spectra of figure 3(*b*) were all taken on cell structures.

Figure 4 refers to a culture with prevailing granule cell population, forming a neuron network. In the course of the experiments, we also found a macrophage cell that was accidentally present in one of the Purkinje cultures. The corresponding data are shown in figure 5 and clearly reveal the presence of Zn in a higher concentration than that of the previous data. This is reasonable, considering the physiological function of macrophages.

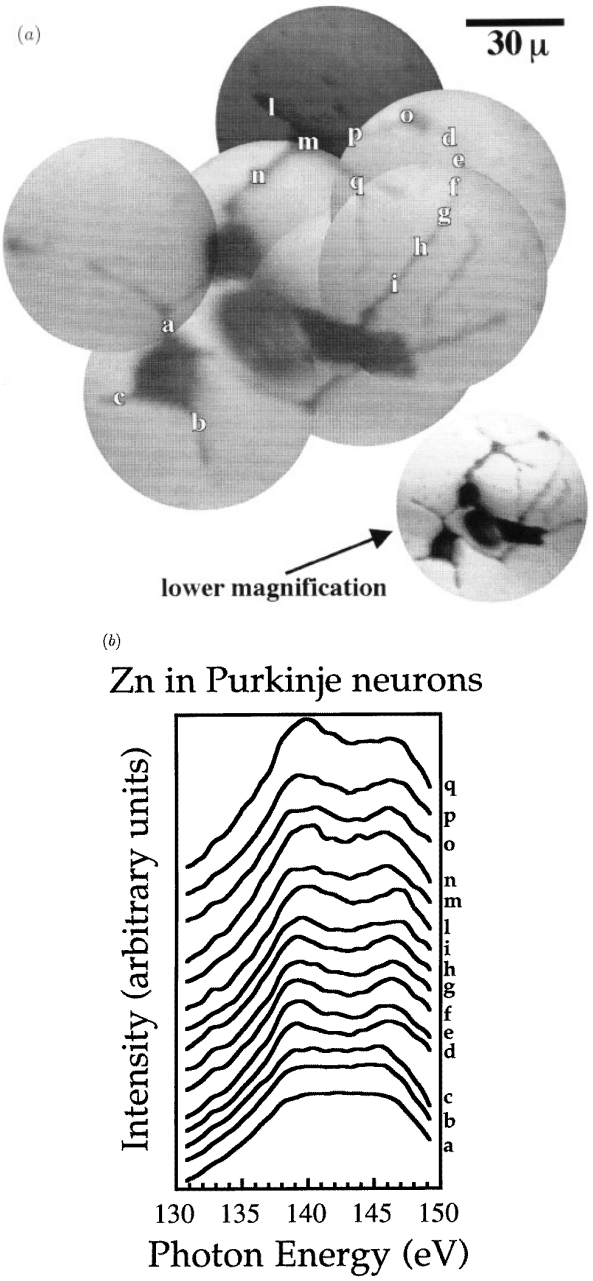


**Figure 3.** Data for a culture with prevailing Purkinje neuron population, exposed for 20 min to a 5 mM $ZnCl_2$ solution.

All of the data in figures 1–5 were taken on cultures that had been exposed to zinc at the end of the growth period. The main issue, of course, was the possible presence of zinc in the cultures prior to exposure. This element, in fact, is a component of zinc binding proteins and is also suspected to be involved in several cell mechanisms such as the regulation of neuronal excitability [17–19].

We addressed this issue by investigating cultures that had not been exposed to zinc. The corresponding data are shown in figure 6. The XSEM spectra of figure 6 confirm our suspicion: zinc is indeed physiologically present in the unexposed cultures. We then addressed the related issue: did the exposure have any effect at all on the amount of zinc

**Table 1.** ICP results normalized to $1.2 \times 10^6$ cells in 1 ml of $HNO_3$ solution; Glu=glutamate 100 $\mu$M; Ka=kainate 100 $\mu$M; $S^=$ =a solution of $Na_2S$, used to induce precipitation of zinc as sulphide. The fixative, when present, was a 4% solution of para-formaldehyde in PBSS. In order to estimate the Zn concentration in the cells, consider that the volume of $1.2 \times 10^6$ cells is approximately 1 $\mu$l. Therefore, 0.1 $\mu$g ml$^{-1}$ of Zn in the conditions of this table corresponds to 100 $\mu$g ml$^{-1}$ in the living cells.

| $ZnCl_2$ concentration (mM) | Exposure time (min) | Neurotoxin or precipitating agent | Fixative? | Days *in vitro* | Detected Zn concentration ($\mu$g ml$^{-1}$) ($\pm$0.1 $\mu$g ml$^{-1}$) |
|---|---|---|---|---|---|
| | 0 | | NO | 8 | 0.2 |
| 1 | 20 | | NO | 8 | 0.3 |
| 5 | 20 | | NO | 8 | 0.6 |
| | 0 | | YES | 7 | 0.2 |
| 1 | 20 | | YES | 7 | 0.2 |
| 5 | 20 | | YES | 7 | 0.4 |
| | 0 | $S^=$ | YES | 7 | 0.2 |
| 1 | 20 | $S^=$ | YES | 7 | 0.3 |
| 5 | 20 | $S^=$ | YES | 7 | 0.4 |
| 1 | 20 | Ka | NO | 8 | 0.6 |
| 5 | 20 | Ka | NO | 8 | 1.2 |
| 1 | 20 | Glu | NO | 8 | 0.3 |
| 5 | 20 | Glu | NO | 8 | 0.8 |

present in the cultures? We addressed this second issue by analysing the zinc concentration in different cultures after different types of zinc exposures. The results, obtained with the ICP–AES technique, are summarized in table 1.

We performed ICP-AES experiments only on granule cells. The density (number of cells per unit area) of these cells in culture is very reproducible. The results of a quantitative analysis of the effects of different exposures on identical cultures can therefore be compared. In the case of glial cells, which undergo mytosis, the density varies from culture to culture, and a quantitative analysis would be affected by population variations. In the case of Purkinje neurons such a variation is even more dramatic. The new culture procedure we used is still being optimized and, in most cases, produces one or two cells per culture, so that again no quantitative comparative analysis is possible on multiple samples.

## 4. Comparisons and discussion

The data of figures 1–6 and of table 1 lead us to the following straightforward conclusions: (1) the exposure does not significantly affect the zinc concentration in the region probed by spectromicroscopy—the surface—, but (2) it does increase the overall zinc concentration. Thus, the effect of exposure is primarily a bulk phenomenon.

The first conclusion can be derived from the spectra of figures 1(*b*)–5(*b*) (Zn-exposed cultures), compared to those of figure 6 (unexposed cultures). We see no significant difference in the zinc signal level or level to background intensity. The data of figure 6 are consistent with many other spectra taken on different cells of unexposed cultures, with prevailinig glial cell, granule cell and Purkinje neuron populations. Furthermore, cells in the cultures were decapped using the procedure described in [20]. This produced portions of cell bodies in which the top membrane had been removed, without significantly affecting the XSEM zinc signal level.

We also note that data similar to those of figure 4 (granule cells) were obtained in cultures of this type, exposed to zinc solution with the simultaneous presence of an excitatory amino acid, kainate or glutamate. In some cases, a precipitating agent, $Na_2S$, was also used. None of these factors significantly changed the zinc signal level; it remained similar to that of unexposed cultures.

These findings are in striking contrast with the ICP-AES data of table 1. First of all, we see a clear tendency to an increase in concentration for increasing exposure concentrations. Note that almost all of these increases are well beyond the experimental uncertainty.

Table 1 does not reveal any significant influence of the aforementioned fixation process on the zinc concentration. Note that fixation guarantees the chemical stability of the sample. Since fixation does not induce Zn affinity, the fixed and dehydrated samples used in the XSEM experiments can be considered chemically equivalent to the unfixed wet cells of the ICP experiments.

The precipitating agent ($S^=$) did not seem to influence the Zn concentration, either. On the other hand, the presence of the excitatory amino acid kainate did seem to influence such a concentration, for example by bringing it from 0.6 $\mu$g ml$^{-1}$ (for exposure with no excitatory amino acids, no precipitating agent and no fixation) to double this value. The data on glutamate suggest a similar, although weaker, effect.

Note that the detection of kainate-induced effects is again in contrast to the spectromicroscopy results, which revealed no such effect. In summary, there is a striking contrast between the spectromicroscopy data and the ICP-AES results, both concerning the exposure with no other factors and the exposure in the presence of excitatory amino

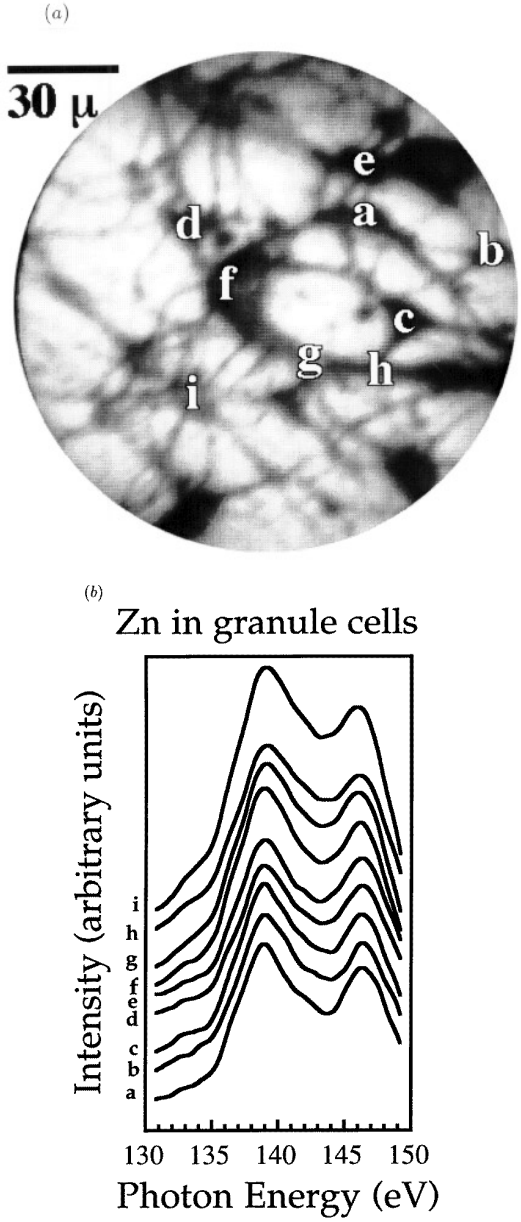


**Figure 4.** Data for a culture with prevailing granule cell population, exposed for 20 min to a 5 mM $ZnCl_2$ solution.

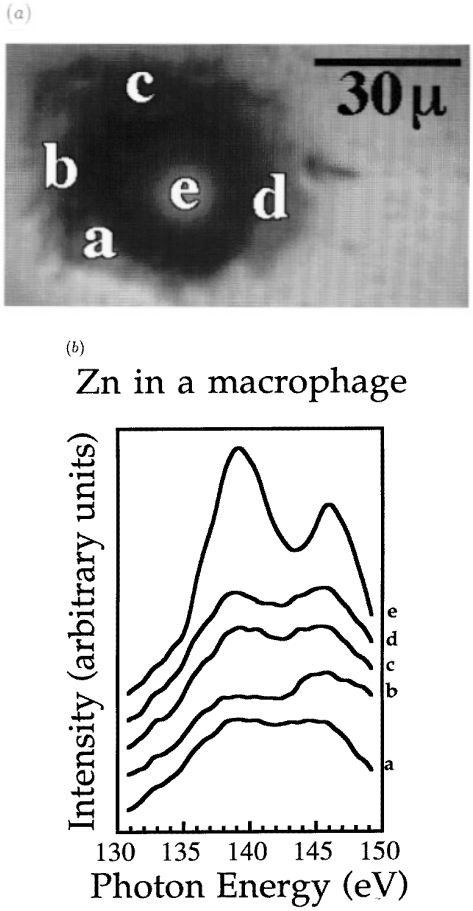


**Figure 5.** Data for a macrophage that was accidentally present in one of the cultures with prevailing Purkinje neuron population. The spectra clearly reveal the presence of Zn in higher concentration than that of the previous data.

acids. These conclusions were consistently confirmed by data taken on at least five different biological preparations for each type of culture and Zn exposure (1 or 5 mM). Furthermore, for each specimen, images were taken at several tens of locations, and spectra on 10–20 spots of each image. The data explicitly presented here are thus representative of a much larger database, and are fully consistent with it. No significant spread was detectable in the Zn3s signal levels or signal to background levels over the entire database.

The main difference between spectromicroscopy and ICP-AES is surface sensitivity. Photoelecron spectromicroscopy is indeed surface sensitive, because it is based on the detection of mainly secondary photoelectrons [7, 8]. Their escape depth [7] from a condensed system is not as short as that of primary photoelectrons, which is determined by the mean free path for inelastic scattering. Secondary photoelectrons are, by definition, produced by inelastic scattering events, and their escape depth is determined by the condition that the final energy of a multiple scattering sequence be above the vacuum level. Furthermore, secondary photoelectrons are mostly emitted at low energies, for which even the inelastic scattering mean free path is large.

As a result, the escape depth for secondary photoelectrons is at least one order of magnitude larger than the minimum primary photoelectron value of 5–10 Å [7]. Even so, a technique like XSEM remains highly surface sensitive, certainly much more than ICP-AES which is not surface sensitive at all. We estimate the thickness of the region explored by our spectromicroscopy investigation to be roughly comparable to the thickness of the membrane region plus the membrane proteins and channels.

The most plausible explanation for the differences between our XSEM and ICP-AES data is, therefore, the fact that the former investigates the surface of our specimens whereas the latter investigates their bulk—where surface and bulk in the present case mean the cell's membrane and its cytoplasm repectively. Our data indicate that Zn is physiologically present, independent of any exposure,

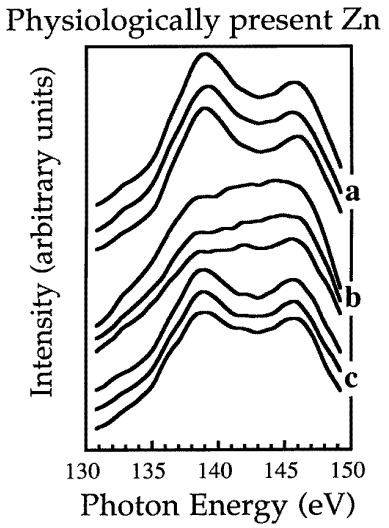


**Figure 6.** XSEM curves from three different cultures with: curves a, prevailing granule population; b, prevailing glial cell population; and c, prevailing Purkinje population. None of the cultures was artificially exposed to Zn solution, therefore the spectra certainly reflect the physiologically present zinc. The cultures a and b were decapped (see [20] for a description of the technique), so the corresponding curves in this figure reflect both the top membrane region and deeper cell regions.

both in the membrane and in the cytoplasm. This is not surprising, for example in the light of the existence of Zn binding proteins.

The differences between XSEM and ICP-AES data, on the other hand, indicate that the artificial Zn exposures only significantly increase Zn content in the cytoplasm. This could be either due to a large physiological amount of Zn in the membrane, which is only marginally affected by the exposure, or to a more effective mechanism for exposure-caused uptake in the cytoplasm—or else to a combination of both effects. Our results cannot definitely rule out any one of these effects. Speculatively, we tend to favour the first conjecture, because of the hypothesised involvement of Zn in synaptic transmission [21]. Spectromicroscopy experiments on decapped cells to detect zinc presence and chemical status in the cytoplasm are the subject of further investigations.

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