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Metal uptake in neurone cultures: a systematic study

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We present the first comparative study of the uptake of metal ions by neurones, performed for Zn, Cr, Co, Mo, Al, Ni, Mn and Cd. The study reveals substantial differences in the uptake of different metals, under similar exposure procedures. In particular, we found very large uptakes for aluminium and molybdenum. We also found significant effects of excitatory substances, in particular kainate, as stimulants of uptake of some of the metals.

Key words: Metal uptake; Toxic elements; Trace elements; ICP (Inductively Coupled Plasma); Photoemission; Spectromicroscopy

Introduction

The uptake of elements in neurones is one of the most active research areas in the life sciences. Particular attention[1–3] has been devoted to metals, because of their toxicity and other effects. Previous investigations, however, have treated individual elements, without a comparative study of different metals. We present the first results of such a study, that was performed with Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP)[4–7] and with a novel technique, photoelectron spectromicroscopy.[8–11] The study concerns the uptake in neurones of eight different metals: chromium, cobalt, manganese, zinc, nickel, aluminium, molybdenum and cadmium. Systematic experiments were performed varying the metal ion concentration and the exposure time.

The study included a comparison of the effects of different excitatory substances in inducing metal uptake. These substances are supposed to excite and swell the neurones, and facilitate the penetration of toxic elements through the ion channels in cell membranes. The most important conclusions are that significant quantitative differences exist between the uptake of different metals under identical conditions, and that, for some metals, kainate enhances the uptake. Specifically, we found a much larger uptake of aluminium and molybdenum than of the other elements. Kainate does not significantly increase the uptake of Al and Mo; it does, however, enhance that of cobalt by a factor of six and that of zinc by a a factor of two.[1,3]

Photoemission spectromicroscopy was used for preliminary qualitative tests of the homogeneity of the metal distributions in neurones. We found significant inhomogeneities when these tests were performed for cobalt and manganese. The remainder of this article will present the experimental procedure in the next section and the ICP and photoemission spectromicroscopy results in the following two sections. The article is concluded by a summary of our conclusions.

Materials and Methods

The specimens were prepared with either one of two different procedures. The first one was used for exposure to Zn, Cr, Co or Mn. Cerebellar granule cells from 7 day old rats were seeded on Petri dishes (approximately 1.2×10^6 cells per dish), treated with 5 $\mu g\,ml^{-1}$ poly-L-lysine solution. The cells were obtained[12–15] by enzymatical and mechanical dissociation of the cerebellar tissue and plated in Basal Medium (Eagle's salt), containing 10% foetal calf serum, and allowed to grow in an incubator at 37°C in a 5% CO_2 humidified atmosphere. After 4 or 6 days *in vitro* depending on the specimen, the cultures were exposed for approximately 17 h to a 5 μM solution of the relevant metal chloride ($MnCl_2$, $CoCl_2$, $CrCl_2$ or $ZnCl_2$) in the culture medium. For each salt, we compared the results obtained with no excitatory substances with those obtained with a mixture of three different substances, kainate, glutamate and *N*-methyl-D-aspartate (NMDA), 100 μM each. These substances are supposed to excite and swell the neurones, and facilitate the penetration of toxic elements through sodium and calcium membrane channels. After 17 h, we removed the culture medium, and exposed the specimens to a solution of the same chlor-

ides (5 mM) in an uptake buffer for 15 min. The uptake buffer composition was: 139 mM sucrose, 57.5 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 12 mM glucose, 10 mM HEPES with a pH of 7.2. The final pH of the buffer was 7.5. At this point, we used a precipitating agent: α-nitrose-β-naphthol to induce precipitation of cobalt, or a solution of Na_2S plus NH_4Cl to induce precipitation of Zn, Cr or Mn sulphates. Then, the samples were carefully washed in the uptake buffer to remove the residual metals not uptaken by neurones. For the ICP analysis, the specimens were suspended in HNO_3. The total age was 5 or 7 days.

The specimens obtained with the first procedure will be identified hereafter with the following code names, that specify the final age of the cells and the presence or absence of excitatory amino acids: 5NN (5 days, no excitatory substances); 7NN (7 days, no excitatory substances); 5NM (5 days, with a mixture of the three excitatory amino acids) and 7NM (7 days, with the same mixture). The second procedure was used for Zn, Cr, Co, Mo, Al, Ni and Cd, i.e. for all metals considered in the present study except Mn. This procedure differed from the first one in the following aspects. First, the number of cells per dish was higher, 8×10^6. Second, for each metal, we prepared four different kinds of samples: the first type was obtained by exposing for 15 h, with no excitatory amino acids, 7 day old cultures to a 5 μM solution of $ZnCl_2$, $CrCl_2$, $CoCl_2$, $MoCl_5$, AlF_3, $NiCl_2$, or $CdCl_2$ acetate. The three other types were obtained by exposure for 20 min of 8 day old cultures to a 5 mM solution of the relevant metal salt, either in the absence of an excitatory substance, or in the presence of kainate or glutamate. The code names used for the samples obtained with the second procedure were: 8NN for the first type; for the other three types: 8+NN, 8+K and 8+G depending whether the cultures were exposed to the non-excitatory agents, kainate or glutamate.

The nitric acid extracts of the specimens were analysed by ICP after dilution 1:1 in double distilled water. In Table 1 we report the plasma emission wavelength for each element. Note that the ICP technique detects spectral lines from both ions and neutral atoms, but the presence of ions in the discharge is not necessarily related to the presence of the same ions in the specimen. Before performing the ICP measurements for a given element, the instrument was calibrated with a standard 0.5 μg ml^{-1} solution of the same element.

Table 1. ICP calibration wavelengths. The plasma atomic emission wavelength used in the ICP measurements of each element

Metal	Wavelength (nm)
Cobalt	228.62
Zinc	213.86
Chromium	267.71
Manganese	257.61
Cadmium	214.44
Nickel	213.6
Molybdenum	202.03
Aluminium	396.15

Results

ICP results: The numerical results given by the ICP analysis[4–7] are summarized in Table 2. The uncertainties reported as percentage, is the standard deviation over a set of six measurements for each experiment. The following conclusions can be drawn from a comparative examination of this table. First of all, it is quite clear that the uptake of aluminium and molybdenum is much more pronounced than that of the other elements, under similar conditions. It is also evident

Table 2. Summary of the ICP results

Uptaken Metal	Specimen	Metal concentration (μg ml^{-1})	Uncertainty (±, in per cent)
Cobalt	5NN	0.40	4
	5NM	0.63	2
	7NN	0.62	4
	7NM	0.90	1
	8NN	0.02	1
	8+NN	0.09	5
	8+K	0.58	5
	8+G	0.23	3
Zinc	5NN	2.36	1
	5NM	2.02	1
	7NN	14.1	3
	7NM	2.50	1
	8NN	0.14	7
	8+NN	0.74	3
	8+K	1.39	1
	8+G	0.85	1
Chromium	5NN	6.40	2
	5NM	5.05	1
	7NN	5.32	1
	7NM	4.89	1
	8NN	0.01	8
	8+NN	1.81	3
	8+K	1.94	2
	8+G	2.75	1
Manganese	5NN	0.50	1
	5NM	0.66	1
	7NN	0.77	0.5
	7NM	1.15	1
Cadmium	8NN	0.04	3
	8+NN	3.14	1
	8+K	2.02	1
	8+G	1.84	1
Nickel	8NN	0.007	20
	8+NN	0.28	3
	8+K	0.43	1
	8+G	0.22	2
Molybdenum	8NN	0.03	6
	8+NN	15.4	1
	8+K	15.7	1
	8+G	14.8	6
Aluminium	8NN	0.14	8
	8+NN	29.2	1
	8+K	24.8	1
	8+G	28.2	0.5

ICP results normalized to 10^6 cells in 1 ml of solution. 5NN identifies 5 day old cells exposed for 17 h to a 5 μM plus 15 min to a 5 mM solution of the relevant element, with no excitatory amino acids; 7NN 7 days, same exposures, no excitatory amino acids; 5NM 5 days, same exposures, with mixture of excitatory amino acids and 7NM 7 days, same exposures, with mixture. 8NN 8 day old cells exposed for 15 h to 5 μM solution of the relevant element with no excitatory amino acids; 8+NN 8 day old cells exposed for 20 min to 5 mM solution with no excitatory amino acids; 8+NN, 8+K and 8+G exposed to 5 mM solutions for 20 min in the absence of excitatory amino acids or in the presence of kainate or glutamate. Tests performed on the blank HNO_3 (used to suspend the specimens) produced no evidence for the presence of the metals under investigation.

FIG. 1. Photoemission spectromicroscopy microimage, taken with the XSEM instrument, of a small area of a neurone specimen.

that the presence of excitatory agents does not modify the uptake of these elements in a substantial way. On the other hand, we find no evidence of nickel uptake, with or without excitatory amino acids. The uptake of cadmium, chromium, zinc, manganese and cobalt, although not as large as that of aluminium and molybdenum, is quite pronounced. We observe that the excitatory amino acid kainate enhances cobalt uptake by a factor of six, and that of zinc by a factor of two (the zinc result is in agreement with Reference 1, the cobalt result with Reference 3). The mixture of excitatory substances enhanced the manganese uptake by a factor of two.

Photoemission spectromicroscopy: Photoemission spectroscopy has been for many years a leading technique in materials science[16–21] for determining electronic and chemical structures. Its impact in the life sciences has been minimal, because of the lack of spatial resolution. Recently, there has been a substantial evolution in this field, and it has become possible to perform photoemission experiments with lateral resolution as good as 0.1 μm, quite suitable for the study of biological specimens.[9–11] In our present study, we performed preliminary tests using the photoemission spectromicroscopy technique known as XSEM (X-ray Secondary (Electron) Emission Microscopy).[8] In essence, the XSEM produces chemical-contrast images with a resolution of the order of 0.5 μm, and XANES (X-ray Absorption Near-Edge Structure) spectra over specimen areas of a similar size. Figure 1 shows an example of chemical-contrast photoelectron microimage obtained on fixed and dehydrated neurones, grown on a gold substrate. The fixation and dehydration was necessary to make the sample compatible with ultra-high vacuum. Figure 2 shows an example of local photoelectron spectrum in the XANES mode, taken in the spectral region of the cobalt 3p absorption edge. These spectra reveal substantial differences in the cobalt concentration from place to place in the neurone network. This demonstrates the need for more detailed microchemical analyses of this kind in order to understand the effects of toxic metals on the neurone systems. A comparison of spectra taken on samples treated with kainate and glutamate, again shown in Fig. 2, confirms the conclusion based on the ICP data: kainate is more effective in stimulating cobalt uptake. Similar tests were performed on cultures exposed to manganese. Once again we found evidence of inhomogeneities in the metal distribution.[6]

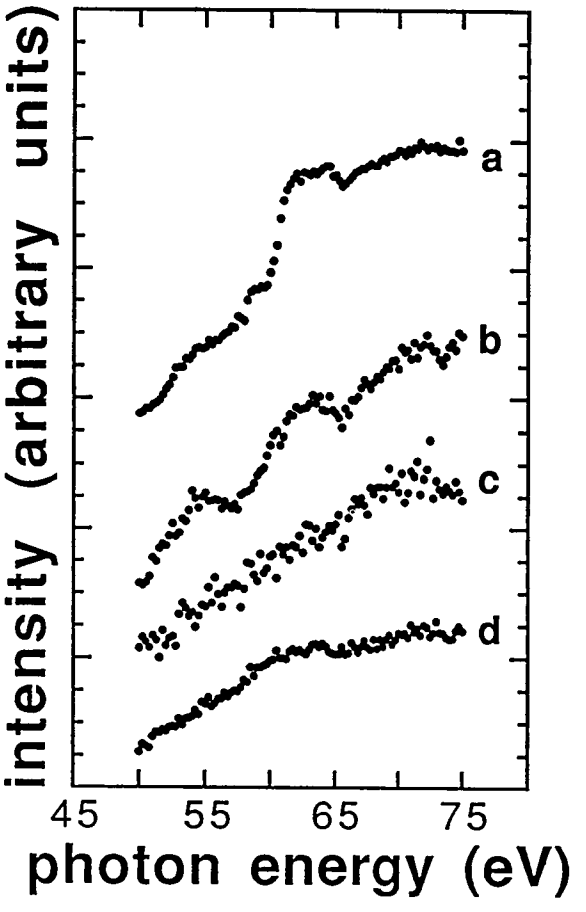

FIG. 2. XANES (X-ray Absorption Near Edge Structure) from several different microscopic (1 μm²) areas of cobalt-doped neurones, revealing spectral 3p features from cobalt. The curves refer to (a) a cobalt wire (taken as reference), (b) an axon area, and (d) a cell body, from a specimen treated with kainate; curve (c) was taken from an axon area of a specimen treated with glutamate.

Conclusions

Our ICP and photoemission spectromicroscopy comparative study of the uptake of different metals under similar conditions has revealed substantial differences from element to element. We have already discussed the quantitative comparisons. We would like to add that no clear correlation exists between the specific class of metal and the magnitude of the uptake: for example, there is no systematic difference between transition and non-transition metals. On the other hand, we confirmed the results described in Reference 3, that kainate is quite effective in promoting the uptake of cobalt. Furthermore, we demonstrated that kainate also promotes the uptake of zinc. This result should be compared with those described in Reference 1, whose conclusions, although derived from a different point of view, are consistent with ours.

Notice, however, that the stimulating action of excitatory substances is by no means universal. We can offer no explanation, at the present time, for their specificity; this is the subject of further studies. The preliminary photoelectron spectromicroscopy results revealed significant inhomogeneities in the metal distribution. This aspect certainly deserves further attention, if the effect of metals in neurones are to be fully understood.

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