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The Effect of Ashing on Cells: Spectromicroscopy of Physiological Elements

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We analyzed the effects of cold oxygen plasma ashing of neurobiological specimens on different elements with synchrotron spectromicroscopy. Our results demonstrate that while carbon is almost completely removed, phosphorus, calcium, potassium, sulfur, and, to some extent, nitrogen are retained and their relative concentration is enhanced.

Ashing is a procedure used to reduce the thickness of tissue sections or to investigate the chemical properties of biological specimens (1–3). This procedure, in particular, is supposed to eliminate certain elements (carbon, nitrogen, and hydrogen) and enhance the relative concentration of others—thus, making it easier to investigate these latter. The effects of ashing on the structure of specimens have been extensively analyzed (1). On the other hand, the chemical effects are much less known.

Our present study analyzes these effects, by comparing ashed and nonashed neurobiological specimens with synchrotron spectromicroscopy (4–8).

MATERIALS AND METHODS

Synchrotron spectromicroscopy yields information on the microchemical composition of the specimen (4–8). It can study biological or materials science samples as long as they are sufficiently conductive, flat, and UHV compatible. The technique consists of sending onto the system monochromatized X rays emitted by a synchrotron light source (the Wisconsin Synchrotron Radiation Center storage ring Aladdin in our case) and detecting the photoelectrons thus produced. The photoelectron intensity vs photon energy curves reproduce the optical absorption coefficient of the system, and from characteristic spectroscopic features one can extract information on the presence and chemical status of each element (9).

We studied ashed and nonashed neurobiological specimens with the recently commissioned MEPHISTO spectromicroscope (from the French acronym "Microscope à Emission de Photoélectrons par Illumination Synchrotronique de Type Onduleur"), with a spatial resolution of 0.2 μm.

The specimens were primary cultures of rat cerebellar granule cells. A detailed description of our specimen preparation process can be found in Refs. (7) and (8). In short, cells extracted from rat cerebellum were allowed to grow for 8 days on gold-coated silicon substrates, pretreated with 10 μg/ml of poly-L-lysine solution. Selective techniques were used to obtain a prevailing population of granule cells (10–12).

At the end of the growth period, the cultures were washed, fixed with paraformaldehyde, and dehydrated. Part of the cultures were analyzed in this form, and others were first ashed with a cold plasma (150 C, Plasma-Processor 300E, Techn. Plasma GmbH, München) in the presence of oxygen for $\geqslant$24 h.

RESULTS AND DISCUSSION

Figure 1 shows an ashed granule cell culture imaged by MEPHISTO. In these micrographs the contrast is originated by the different photoelectron emission

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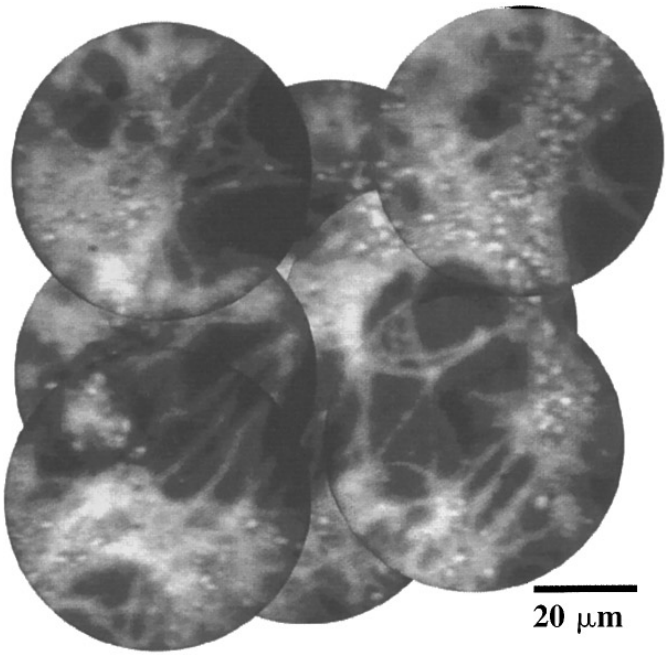

FIG. 1. Patchwork of micrographs of an ashed granule cell culture on a gold-coated silicon substrate. The images were obtained using the recently commissioned MEPHISTO microscope, illuminating the specimen with monochromatic photons of 140 eV.

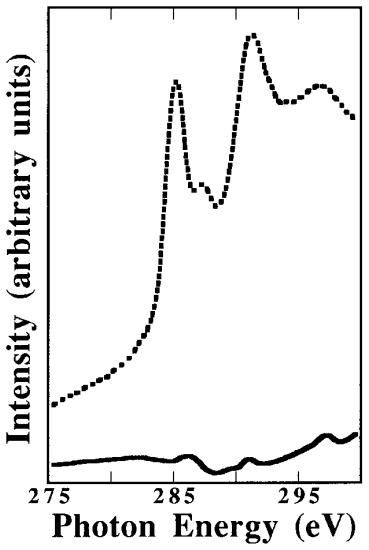

FIG. 2. X-ray absorption curves in the C1s edge spectral region taken on ashed and nonashed neuron cell culture. In this and the following figures, the solid line refers to ashed cells and the dashed line refers to nonashed cells. It is evident that carbon is almost completely removed by ashing. The spot sizes from which the two spectra were taken was $20 \times 20\ \mu m^2$.

yield of different elements. In the present case, the images were acquired at 140 eV photon energy, where phosphorus is photoemitting more electrons than gold; therefore, the phosphorus-containing cells appear brighter than the surrounding gold substrate.

Note that after ashing the cell thickness is reduced and the cell structures appear flat. Carbon constitutes the backbone of the macromolecules supporting the cytoarchitecture of cells; therefore, by removing carbon by ashing, the cells lose their skeleton and consequently their three-dimensional structure.

With the MEPHISTO microscope one can select microscopic areas on micrographs like those of Fig. 1 and acquire X-ray absorption spectra scanning the photon energy, thus revealing the chemical composition of specific features such as cell structures.

Figures 2–8 show results obtained in the spectral regions of the carbon 1s, nitrogen 1s, oxygen 1s, calcium 2p, phosphorus 2p, potassium 2p, and sulfur 2p edges. All spectra are normalized to the monochromator yield curve.

A comparison between the spectra for ashed and nonashed specimens, reported in all figures, shows the results of our study.

Specifically, as shown in Fig. 2, carbon is almost completely removed by ashing. We also note that the traces of carbon left after ashing appear in a different chemical status with respect to the nonashed sample. The first spectral feature, in fact, occurs at 285 eV (aliphatic carbon) before ashing and at 286 eV after ashing. Such a difference may be related to a more oxidized C state formed during ashing with oxygen plasma.

On the other hand, Fig. 3 shows evidence of the pres-

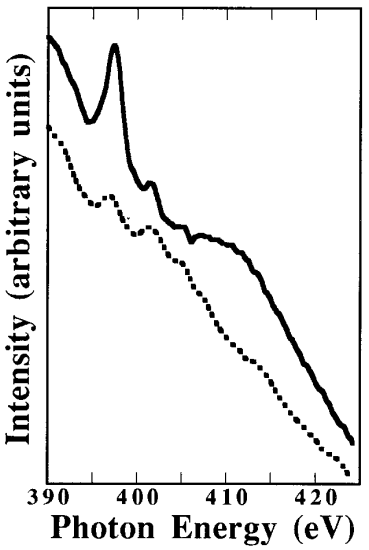

FIG. 3. Similar spectra for the N1s edge spectral region. Note that nitrogen is still clearly present after ashing, although the spectral line shape appears different in the two spectra, suggesting a different chemical status.

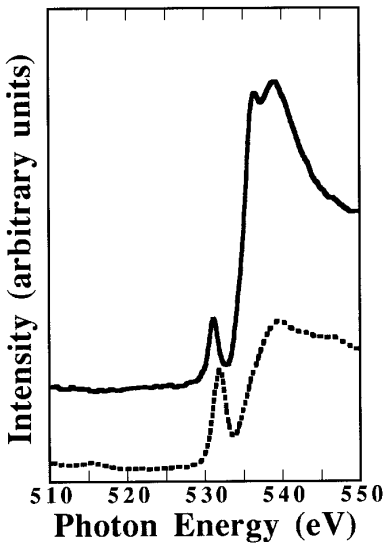

FIG. 4. Similar data for the O1s edge. Note that after ashing the oxygen edge relative intensity is higher, corresponding to a higher concentration of this element, and a new oxygen structure appears at 537 eV.

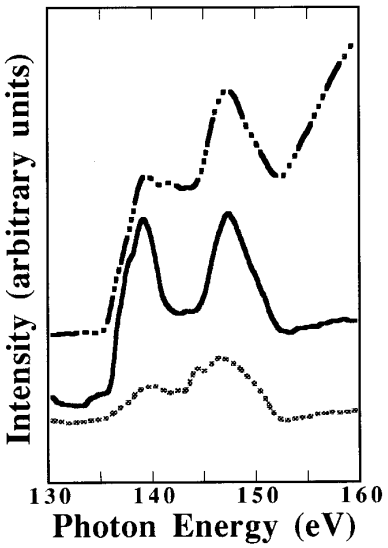

FIG. 6. Spectra of the P2p absorption edge. It is evident that the relative phosphorus concentration is strongly enhanced by ashing. Also shown is a reference spectrum taken on a dried droplet of $FePO_4$ in water (dashed–dotted line).

ence of nitrogen even after ashing, with no significant difference in N concentration. This somewhat surprising result can be explained by the probable differences between the carbon-removal process and the nitrogen-removal process.

The chemical state of N is also modified by ashing: the first peak at 400 eV is more intense than the edge at 403 eV in the ashed sample, and vice versa in the nonashed sample. This feature at 403 eV may correspond to the aminic group $-NH_2$ present in basic amino acids, and $-CONHC-$ group in peptide bonds (13), removed by ashing. Also, the spectral "shoulder" at >410 appearing after ashing can be interpreted as $-ONO_2$, NO_2, and $-ONO$ (13), most likely being formed during the oxidation procedure.

Figure 4 shows that oxygen, as expected, is not removed by the ashing process, and yet its concentration is enhanced. We also observe that ashing induces the

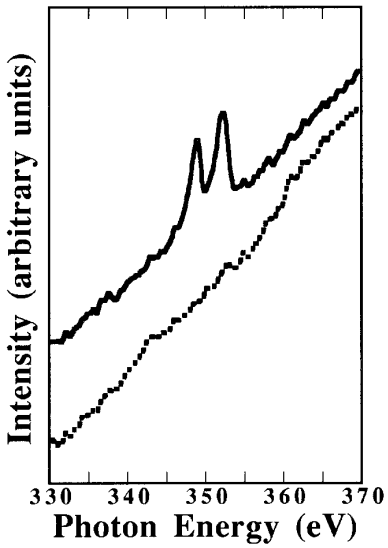

FIG. 5. Similar data for the Ca2p edge spectral region. It is clear that the relative calcium content is strongly enhanced by ashing.

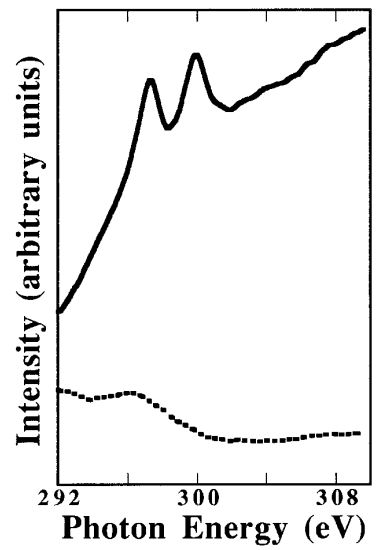

FIG. 7. Spectra of the K2p edge. In this case K becomes detectable only after ashing.

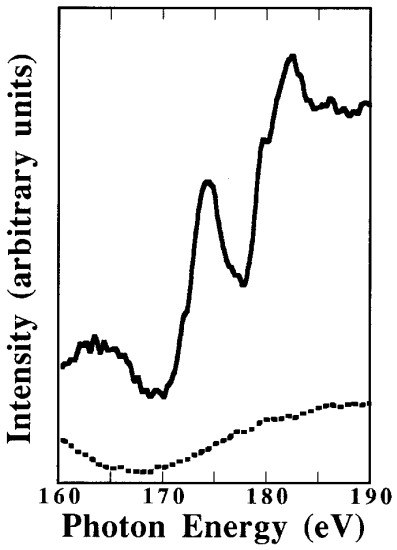

FIG. 8. Data for the S2p edge. As in the case of K and Ca, sulfur is detectable only after ashing.

formation of a new chemical state, corresponding to the spectral feature at 537 eV. This state must be an oxide form, induced by ashing on one of the other elements present in the sample.

The most important point elucidated by our analysis is the enhancement of the calcium, phosphorus, potassium, and sulfur concentrations by ashing.

Specifically, the data of Fig. 5–8 demonstrate that ashing makes it possible to investigate the properties of calcium, potassium, and sulfur in neurobiological specimens, which are of crucial importance for a large variety of fundamental physiological functions. The relative concentration of phosphorus is also enhanced by ashing, even if phosphorus was well detectable even before ashing. Probably, phosphorus is not removed by ashing/oxidation because it occurs as phosphate, and it is therefore already completely oxidized. To test this hypothesis, we took data on a dried droplet of water solution of $FePO_4$; a comparison between one of the corresponding spectra (shown in Fig. 6) and the cell spectra confirms that the oxidation state of P is similar in the two cases. The peaks present in the nonashed sample spectrum of Fig. 6, at 142 and 145 eV are the C1s second-order peaks (at one-half the energy of the same features of Fig. 2).

CONCLUSIONS

We analyzed the effects of cold oxygen plasma ashing on neuron cells by synchrotron spectromicroscopy. By comparing spectra taken on ashed and nonashed cell cultures we conclude that: first, carbon is almost completely eliminated by the ashing process, as expected. Second, we found that nitrogen is not removed, although its chemical status is altered by ashing. Third, we found that the relative concentrations of oxygen, calcium, phosphorous, potassium, and sulfur are dramatically increased. Altogether our results indicate that the ashing procedure applied to biological samples greatly facilitates the study of fundamental elements present in small amounts or trace concentrations.

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