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QUANTITATIVE MICROANALYSIS OF NITROGEN DISTRIBUTION IN CARPOSPORES OF *GRACILARIA SECUNDATA* WITH SCANNING AUGER MICROSCOPY

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Abstract

Quantitative microanalysis has been performed with a JAMP 10 Scanning Auger Microscope at 0.5 μm spot size, on carpospores of *Gracilaria secundata* Harv.. Samples were fixed in glutaraldehyde and osmium tetroxide and embedded in plastic material. Thin sections were placed on solid copper discs. the surfaces of which had been smoothed by electropolishing.

Quantification of the nitrogen content was performed using a standard with known content of nitrogen.

Nitrogen was localized in the nucleus, chloroplasts and osmiophilic inclusions of the carpospore. Trace amounts of nitrogen were also found in cell walls, starch grains and in the embedding material.

The use of Scanning Auger Microscopy on biological material is discussed.

Key words: Scanning Auger Microscopy, red algae, nitrogen, quantitative analysis, biological specimens.

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Introduction

A low nitrogen concentration in seawater is often a limiting factor for the growth of algae. Therefore, many species of marine macroalgae have developed different ways to accumulate and store nitrogen during periods of high availability, such as the winter months, for future use during the spring and summer months when the nitrogen concentration is low. Species of *Laminaria*, for instance, store large amounts as inorganic nitrogen (Chapman and Craigie, 1977) while the red seaweed *Chondrus crispus* stores nitrogen in the form of a dipeptide (Laycock and Craigie, 1977). Accessory pigments located on the thylacoids such as phycoerythrin and phycocyanin are often considered as a nitrogen reserve pool, since nitrogen-starved red seaweeds are often observed to have reduced amounts of these pigments (Lapointe and Ryther, 1979). To our knowledge no other nitrogen pools have yet been localized in red seaweeds at the subcellular level.

The aim of the present study was to localize and quantify nitrogen in the carpospores of the red alga *Gracilaria secundata*. The three analytical techniques which are available for analyzing elements in biological sections with a spatial resolution that makes subcellular localization possible are X-ray microanalysis, electron energy-loss spectroscopy, and Auger electron spectroscopy. The most promising method of these for light elements, in theory, is Auger electron spectroscopy. In practice, however, difficulties such as specimen charging and specimen surface contamination have been associated with the method, when used on biological samples (Hart, 1974; Janssen and Venables, 1979). However, recently promising results on nitrogen mapping in thin sections of plastic embedded biological material mounted on solid copper grids have been reported (Stridh et al., 1986) although no quantitative analysis at subcellular resolution has been carried out.

Material and Methods

Plant material

Gracilaria secundata Harv. was collected in Manukau Harbour, New Zealand, in December 1983 and has since then been kept in unialgal culture. The alga was cultured in 25 l plexiglass cylinders with recirculation of the medium (Lignell et al., 1987). Filtered (0.2 μm) seawater with 3.3% salinity and enriched with ES-medium (Provasoli, 1968) was used as medium. Half of the medium volume was changed weekly. Light was provided continuously by fluorescent tubes (Thorn Polylux 4000) at a photon irradiance of 250 $\mu Em^{-2}s^{-1}$. The temperature was maintained at 25 ± 1°C.

Carposporophytes, which had developed in the culture, were fixed overnight in chilled 2.5% w/w glutaraldehyde buffered with sodium cacodylate buffer at pH 7.2 and containing 1% w/w caffeine and 0.25M sucrose (Mueller and Greenwood, 1978).

Transmission electron microscopy

The material was postfixed with 2% w/w osmium tetroxide at 4°C for 2 hours. Specimens were embedded in Epon and sections were double-stained with lead citrate and uranyl acetate. Ultrastructural examinations were carried out with a JEOL 100B electron microscope at 60 kV accelerating voltage.

Auger microanalysis

Specimens to be used for Auger analysis were postfixed in osmium tetroxide, but not stained. Approximately 40 nm thick sections were placed on solid copper discs the surface of which had been smoothed by electropolishing (Stridh et al., 1986).

The analyses were performed with a JAMP 10 Scanning Auger Microscope in beam brightness modulation (BBM) mode at 10 kV accelerating voltage, 25 nA probe current and about 0.5 μm spot size. The spectra obtained were the average of ten energy scans from 290 to 410 eV. The channel size was 1 eV and the counting time at each channel was 600 ms. Specimen surface contaminations were removed by sputtering with 1 keV Ar^+ ions before analysis.

A standard was used for quantitative determination of the nitrogen content. This standard was prepared by mixing 50% w/w glutaraldehyde with urea (Roos and Barnard, 1984). After polymerization the standard was sectioned and transferred to a solid copper disc. Spectra of the standard were recorded under the same conditions as those used for the seaweed specimens. The nitrogen content of the standard was determined with an elemental analyzer, model Carlo-Erba 1106.

A major requirement for a good standard is that it has composition and density values similar to those of the sample to be quantified. The composition of the present alga specimen was compared to that of the nitrogen standard in Auger spectra between 60 eV and 530 eV (Fig 1). The alga specimen contained somewhat more oxygen but less carbon than the standard. However, the resemblance was fairly close.

Quantitative nitrogen concentration values C_N (in % w/w) for the specimens were calculated according to:

$$C_N = \frac{A_{spec}}{A_{stand}} \cdot C_0$$

were C_0 is the known nitrogen concentration of the standard, and A_{spec} and A_{stand} are the measured areas corresponding to the nitrogen spectrum peaks in the specimen and in the standard respectively. In both cases the spectra display a major peak at 375 eV and a minor peak at 358 eV. The nitrogen peaks are broad and asymmetric, hence it is difficult to distinguish the peak from the background. In order to measure the peak areas A, two different background subtraction methods were employed. According to the first method, the background was defined by a straight line between the local minimum at the high energy side of the peak and a spectrum point at 350 eV at the low energy side. The point p was chosen because of the assumption that the peaks are roughly symmetric around 370 eV. According to the second method the background line was chosen between the local minimum at the high energy side and that on the low energy side of the peak. By this second method also the 'low energy tail' of the peaks was included in the peak areas. In such a spectrum the background lines, as defined above, are close to a tangent to the spectrum at the high energy side of the peak. The areas were cut out from the chart paper and weighed with 0.1 mg accuracy. The density of the record-paper used varied with ±1%.

Results

Transmission electron microscopy

Transmission electron micrographs show that the ultrastructure of a mature carpospore is strictly regular (Fig. 2), with the nucleus at the center of the cell and chloroplasts and starch grains arranged in rays emerging from the nucleus. Osmiophilic inclusions of varying sizes are distributed in the outer part of the cell. Organelles such as the nucleus, the chloroplasts, the starch grains and the osmiophilic inclusions occupy the major part of the cell volume.

Auger microanalysis

In microanalysis, an indispensable feature of the applied technique is that

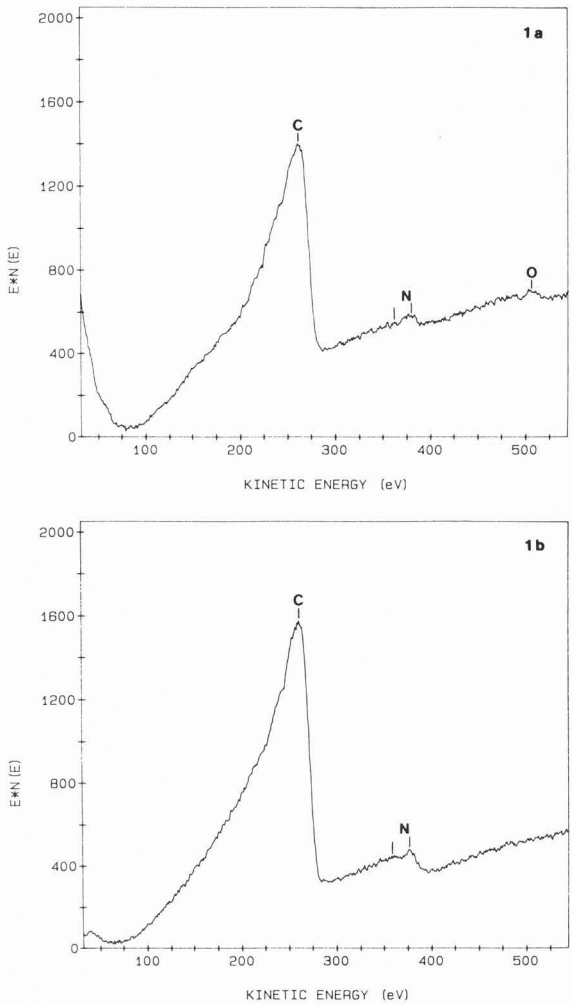
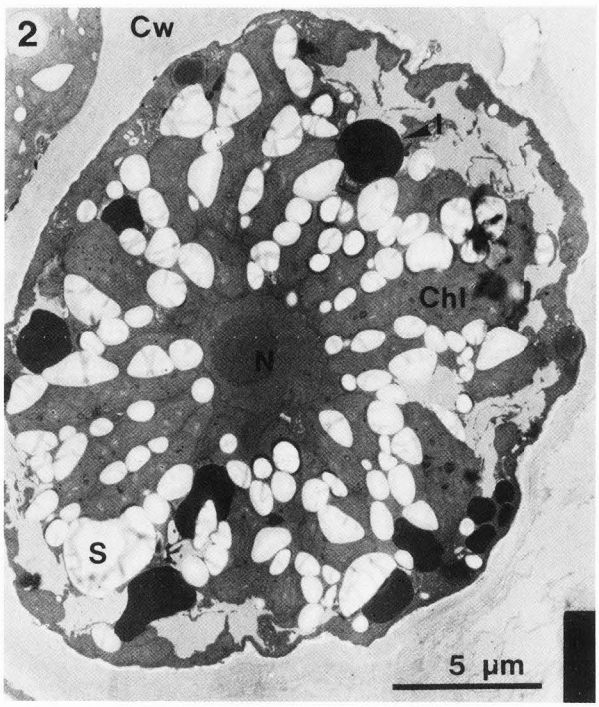


Fig. 1: Auger spectra recorded from a nucleus in an alga specimen (1a) and from the nitrogen standard (1b). The spectra were obtained with an accelerating voltage of 10 kV, 25 nA probe current, 0.5 μm spot size, channel size of 1 eV and counting time at each channel of 600 ms.

it allows the choice of microarea by positioning the electron probe in an image of the specimen. In the present case organelles were visible and could be identified in emissive mode images, although the contrast was low and the resolution somewhat poor (see Fig. 3).

Fig. 4 shows typical Auger spectra obtained from the cell organelles as well as from the cell wall and the plastic embedding. Trace amounts of nitrogen were found even in the plastic material, probably due to atmospheric nitrogen trapped during the polymerization, or as surface contamination during the analysis. The highest amount of nitrogen was found in the nucleus (Table 1) followed by the chloroplasts. The nitrogen concentration



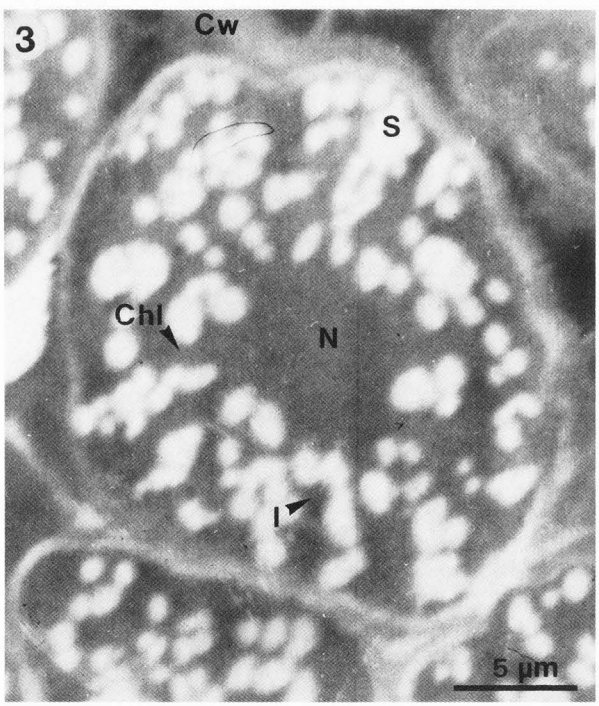


Fig. 2: A transmission electron micrograph of a carpospore of *Gracilaria secundata*. N = nucleus, Chl = chloroplast, I = osmiophilic inclusion, S = starch grain, and Cw = cell wall.

Fig. 3: A secondary electron micrograph of a carpospore of *Gracilaria secundata*. N = nucleus, Chl = chloroplast, I = osmiophilic inclusion, S = starch grain, and Cw = cell wall.

Table 1

Concentrations of nitrogen in cell organelles in carpospores of Gracilaria secundata calculated by two different approximations employed for background subtraction, as described in the section Auger microanalysis. Mean peak area (A_1, A_2), concentration of nitrogen in % w/w (C_1, C_2), standard deviation (σ), number of measurements (n).

	A_1	σ	A_2	σ	n	C_1	σ	C_2	σ	n
Nucleus	329	±71	845	±266	5	16	±3	18	±5	
Chloroplasts	238	±64	552	±146	4	11	±3	12	±3	
Osmiophilic bodies	275	±115	564	±263	8	13	±5	12	±5	
Starch grains	76	±37	130	±93	4	4	±2	3	±1	
Cell walls	94	±13	140	±47	5	4	±2	3	±2	
Plastic embedding	75	±36	136	±75	5	4	±1	3	±1	
Nitrogen standard	503	±25	1126	±150	5	23.7	±0.6			3

of the osmiophilic inclusions varied considerably. Some contained only low amounts of nitrogen, whereas others had very high amounts. Only small or even trace amounts of nitrogen were found in the starch grains and in the cell walls. It can be argued whether these values are within the accuracy of the method or not.

The nitrogen concentrations were measured with the two methods of background subtraction described in the section on **Auger microanalysis** above. The difference in concentrations obtained with the methods was small, although the values obtained with the first approximation were slightly lower in organelles with a high nitrogen content. The values are given in Table 1, in % w/w of the plastic embedded specimen. Since the density of the plastic material is 1.17 g/cm^3, compared to 1 g/cm^3 in the fresh alga, the nitrogen concentration values of Table 1 in fresh alga are underestimated by $\leq$ 17%.

No specimens showed any damage or disturbance due to heating or charging during the Auger analysis.

Discussion

The results show that it is possible to use Scanning Auger Microscopy to detect and analyze nitrogen at the subcellular level in thin sections of biological material. The specimen to be analyzed must, however, give rise to good enough contrast in the emissive mode image to allow the identification of the different cell organelles. In experiments with sections of freeze-substituted unstained carpospores (unpublished results), the contrast was too low to visualize the organelles in the Scanning Auger Microscope. Cryo-prepared specimens might present an advantage, compared to conventionally fixed and dehydrated specimens, because of less risk for diffusion of compounds during the preparation procedure. However, most of the nitrogen in *Gracilaria* species is integrated in proteins, free amino acids and nucleotids, compounds which are fixed by glutaraldehyde, and only a minor part exists in the form of inorganic nitrogen as NH_4^+ or NO_3^- (Bird et al. 1982; Rosenberg and Ramus, 1982). Nitrogen lost due to ion diffusion during preparation thus should correspond to a very small part of the total nitrogen content of the specimen.

Light elements, such as nitrogen, may also be lost from the specimen during Auger microanalysis (Janssen and Venables, 1979). In an experiment (unpublished results) on the nitrogen standard with a probe current of 500 nA (to be compared with the probe current of 25 nA used in this study) a quantity of 12% w/w nitrogen was estimated from a derivate Auger spectrum (see Handbook for Auger Electron Microscopy, 1982) as compared to the actual nitrogen content of 23.7% w/w. This shows that nitrogen may be lost at high currents. However, since the probe current in the present study was very low, it is reasonable to assume that the loss of nitrogen was small and of the same order of magnitude in the specimens as in the nitrogen standard.

Background subtraction from broad, asymmetric peaks like those of the present spectra, is a well known problem, to which no general solution has been worked out. Therefore, absolute quantitative concentration measurements are hardly meaningful. Relative measurements against a suitable standard, however, are rather insensitive to the choice of background level, as this investigation shows, and the obtained nitrogen concentration values can be accepted with reasonable confidence. The difference between the two

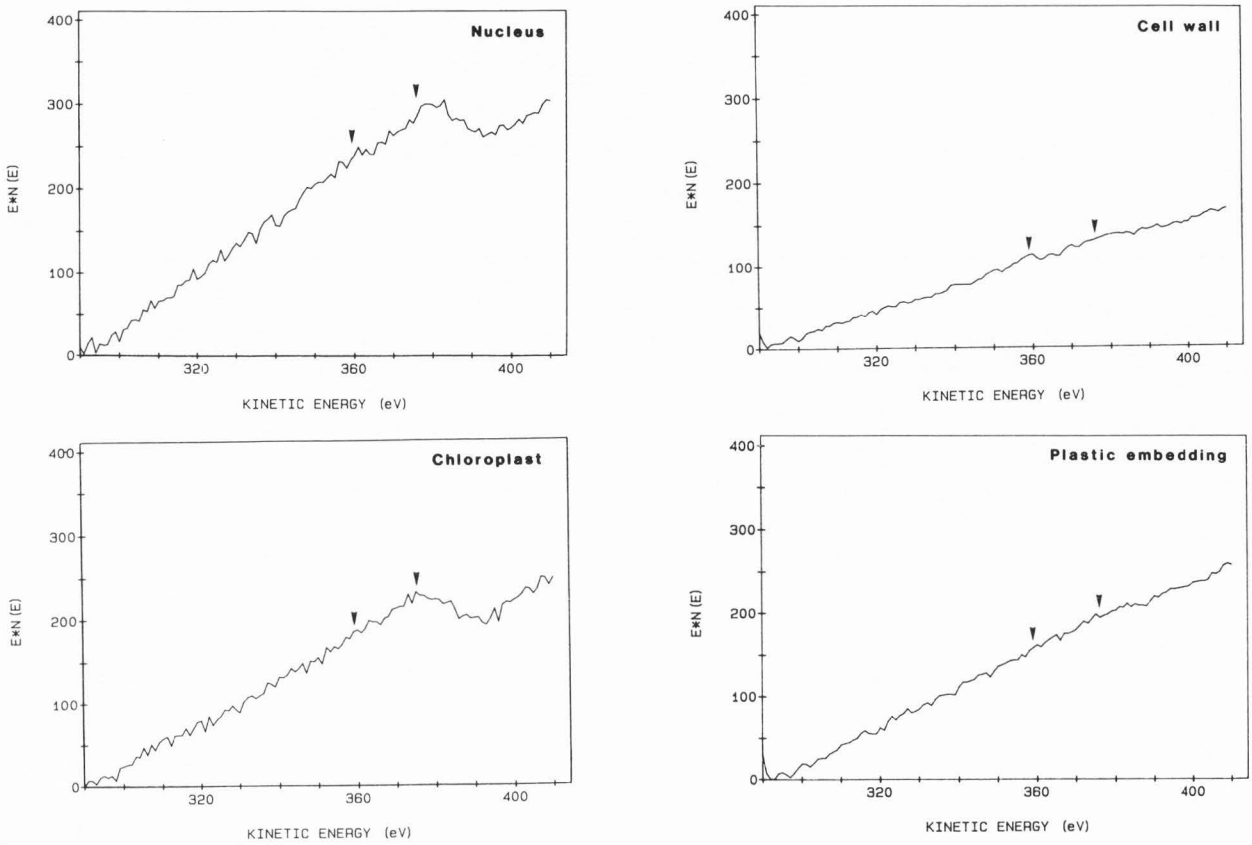


Fig. 4: Parts of Auger spectra, showing the nitrogen peaks, obtained from different cell organelles in carpospores of *Gracilaria secundata*. The Auger spectra were obtained in the absolute, E*N(E), mode. The 358 and 375 eV lines are indicated by arrows.

approximations is small and within the scatter of the methods.

There were significant differences in nitrogen content between different cell organelles. The great variation in nitrogen content of the osmiophilic inclusions may reflect the varying nitrogen content of inclusions of different origin. In the literature these structures have been reported to contain lipids and aldehydes (Feldmann and Feldmann, 1950), phenols and proteins (Young, 1978), bromoterpenes (Young et al., 1980) and polyphosphates (Kugrens and Delivopoulos, 1986). X-ray microanalysis has been used to demonstrate bromine and iodine in the osmiophilic inclusions of some of the red seaweeds (Young et al. 1980, Pedersén et al. 1980). The bromine is probably stored in the form of brominated phenols (Pedersén et al., 1974). In the present case the osmiophilic inclusions of the carpospores also show similarities to the cyanophycine granules of cyanobacteria, which contain both arginine and aspartic acid and function as

a nitrogen store (Simon, 1971). The observed nitrogen variation may also in part be explained by the small size of the inclusions, which are close to the spatial resolution of the method, and by the difficulties to identify the inclusions in the emissive mode image. Part of the area surrounding the inclusions thus could have been included in some of the analyses. The surrounding areas consist mostly of chloroplasts and starch grains, which were found to have a similar or lower nitrogen concentration, respectively, than the inclusions. Therefore, the estimated concentration values for the inclusions might be slightly underestimated.

In general, the nitrogen content of proteins is in the order of 20-25%. Depending on the density of the structures analyzed and the amount of plastic that has pervaded the protein matrix, the actually measured N content would be expected to be lower than 20%.

The present analysis shows that a major part of the total content of nitrogen in the carpospores of *G. secundata* is located in the chloroplasts and the nucleus. Significant amounts are also located in some of the osmiophilic inclusions of the cytoplasm. It remains to be elucidated, however, in what form the nitrogen is stored in the inclusions. It is possible that the nitrogen content could vary with growth conditions and age of the plant. The name **osmiophilic inclusions** only refers to the osmiophilic appearance. Probably, different and variable classes of these inclusions exist.

To our knowledge, this is the first report of the use of Scanning Auger Microanalysis for subcellular localization of elements for quantitative elemental analysis, and, as such, this study represents a break-through. There is a lack of previous experience in the field. The present work should be regarded as a preliminary study, and a more rigorous evaluation of preparative methods and an analytical conditions is needed. Because of the demanding requirements on adequate biological problems, the difficulties in specimen preparation and the complexity of instrumentation for Auger analysis, quantitation included, cooperation between biologists and physicists seems a necessity in this kind of study.

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Discussion with Reviewers

D.G. Castner: Could you provide plots of the measured nitrogen content vs probe current and vs exposure time at each probe current?
P.H. Holloway: The assumption that the loss of nitrogen was small could easily have been checked. Were there any losses? Both samples? How much?
Authors: Loss of mass and of specific elements during electron beam irradiation is not a problem specific for Auger microanalysis and we are well aware of this complication. A detailed study of nitrogen loss from biological specimens during Auger microanalysis would require a separate study. It should also be noted that specimen and standard were of a similar chemical composition (cross-linked protein matrices) and that they were analyzed with the same probe current and exposure time. It would seem reasonable that nitrogen loss in specimen and standard is comparable and, since the nitrogen content in the specimen is calculated relative to that in the standard, the effect of nitrogen loss on our quantitative results may well be negligible. However, we agree that as a matter of principle, it would be interesting to carry out the study requested by the reviewers.

D.G. Castner: Sectioning could result in smearing of material from one region to another region in the cell. Could you show evidence of how the surface composition either changes or remains constant during the sample preparation processes?
Authors: The study was carried out on aldehyde-fixed samples. Soluble material is displaced and lost during the fixation and dehydration steps, and only fixed material remains. It is unlikely that fixed proteins are displaced during the sectioning step.

D.G. Castner: No evidence is given to show how the bulk and surface compositions of the sample are related. This is particularly important since both the standards and the samples are heterogeneous materials. Please comment.
Authors: The standard is homogeneous at the level of analytical resolution. In the specimen, separate cell compartments were analyzed in sectioned material. Although the surface of the sections was analyzed, these surfaces actually represent cross sections at various levels through the compartments of interest. Inhomogeneity within a particular cell compartment will be reflected in the spread of the measurements, rather than in the mean value.

D.G. Castner: Why are broad Auger peaks with large low energy tails observed? Is this typical of instrumental performance? Is this peak shape observed on well-defined conducting samples? Is it due to charging or inelastic losses?
D.C. Sigee: It is very difficult to distinguish the major and minor nitrogen peaks in the Auger spectra shown (figs. 1 and 4). Is this a general feature of Auger analysis of biological material and does the identification of an element by this technique require the quantitative subtraction of background material?
Authors: The shape of the Auger spectrum is typical for the performance of the instrument in the E*N(E) mode (absolute spectra) for specimens containing relatively low elemental concentrations. In the derivative form (dN/dE mode) it is somewhat easier to identify peaks, but this mode it not suitable for quantitative analysis as carried out in this paper. The form of the spectrum has nothing to do with the conductive properties of the specimen.

D.C. Sigee: What are the quantitative limits of elemental detection in biological material by Auger analysis?
Authors: There is insufficient experience with Auger microanalysis of biological specimens to give a definite answer.

However, for inorganic specimens under optimal conditions, the minimal detectable concentration is generally given as 0.1 %. It is unlikely that these optimal conditions can be reached in the analysis of biological specimens.

P.H. Holloway: Were full surveys done and any other elements seen besides C, O, and N?
Authors: No other elements besides C, O and N were detected, and such elements, if present, would therefore be expected to occur only in low quantities (below the detection limit). It should be taken into account that the analysis was carried out on fixed material from which soluble ions such as potassium, chloride and phosphate were lost.

D.C. Sigee: What proportion of nitrogen present in the different cell locations could be attributed to the plastic embedding medium?
Authors: As Table 1 shows, the concentration of N in the plastic could be as high as 4%. To make an accurate correction, one would need to know the local concentration of plastic in the compartments analyzed.

P.H. Holloway: What is a Carlo-Erda 1106 elemental analyzer and on what basis does it operate?
Authors: After combustion of the specimen, the resulting gas is analyzed by gas chromatography.