In order to survive, a tolerant organism must have a mechanism to prevent toxic elements from affecting its metabolism, or damaging the sensitive structures within the cells, e.g., membranes and cell organelles. Recently, phytoplankton organisms isolated from lakes contaminated with copper and nickel (typically 0.5 ppm copper and 2.5 ppm nickel) in the Sudbury, Ontario region, have been shown to be copper and nickel tolerant (14). Related algae from laboratory stocks, used for comparison, were far less tolerant. For two species of the green alga *Scenedesmus*, copper was toxic at 0.15 ppm and 1.5 ppm for the nontolerant and the tolerant strains, respectively.

Copper uptake occurred in both the tolerant and the nontolerant cells (15). The amount taken up was proportional to the amount in solution over the ranges tested: for example, the tolerant...
Scenedesmus grown in media with initial copper concentration of 1.0 ppm had 7,600 μg copper/gram dry weight of algae at day 6, while with 0.5 ppm copper in the medium, the concentration at day 6 was only 3,700 μg copper/gram dry weight.

Since the non-tolerant Scenedesmus failed to grow in media with 0.15 ppm copper or higher, these high levels of copper in the alga were never attained. Other factors such as age of cells and aeration of cultures may vary, but it is clear that the tolerant cells do not exclude copper from the cells but rather accumulate high levels of it, while growth and cell division continue. Copper, like some other heavy metals, actively interacts with free sulfhydryl groups, and therefore may be an important inhibitor of some sulfhydryl-dependent enzymes. Thus, the ability of the tolerant Scenedesmus strain to overcome the deleterious influence of a toxic metal suggests the presence of a defense mechanism to accommodate against environmental toxicity.

The purpose of the present study was to determine by electron microscopy and the application of energy dispersive X-ray (EDX) microanalysis the site of copper accumulation in the cells and to identify any changes in fine structure as a result of copper treatment. The EDX analysis system makes possible an element analysis of chosen cellular regions.

MATERIALS AND METHODS

The algae selected for the study were Scenedesmus acuminatus (Lag.) Chodat 1902 (lab strain, non-tolerant) and Scenedesmus acutiformis var. alternans Schröder 1897 (tolerant strain, referred to as “B-4”). The cells were grown in batch culture in modified Bold’s basal medium (14) without chelate at 25°C in a 16-h light/8-h dark diurnal cycle for 6 days, at which time the cultures were in exponential growth and were harvested as described previously (14). Copper as copper sulfate was added to individual flasks of medium to give the appropriate concentrations of the metal.

For electron microscopy, cells were fixed in 2.5% glutaraldehyde buffered with 0.1 M phosphate buffer, pH 7.2, for 3 h at room temperature. This was followed by postfixation in 2% osmium tetroxide in the same buffer for 2 h at 4°C. The cells were dehydrated in a graded ethanol series and embedded in Spurr’s low-viscosity resin (12). Unstained and lead-counterstained sections were viewed in a Siemens 101 electron microscope at 80 kV. Sections mounted on carbon-coated nylon grids were lightly covered with vaporized carbon and observed with a Philips transmission electron microscope fitted with a Model 606 X-ray spectrometer, a Model 707A energy dispersive analysis of X-rays system and an EDIT system from EDAX International Inc., Prairie View, Ill. Analyses were performed at 80 kV with a total beam current of 50 μA operated at 20 eV per channel. Usually, but not invariably, the spot or probe sizes ranged from 0.32 to 0.2 μm in diameter. X-ray spectra were obtained over the structures specifically investigated in this study and, for comparison, over adjacent areas taken at random.

RESULTS

The general ultrastructural appearance of Scenedesmus B-4 cells cultured in 0.1 ppm copper was relatively normal except for focally increased vacuolation in the cytoplasm. These vacuoles appeared optically empty and structureless, and little or no accumulation of the metal ions could be detected. Occasionally, plastidial accumulation of starch was noted. In cells exposed to 0.7 ppm copper, the cytoplasm contained abundant dense bodies, lipid droplets, and numerous interlacing vacuoles occupied by electron-opaque precipitates of the metal. The lab strain Scenedesmus at low levels of copper, 0.025 ppm, showed copper distributed randomly throughout the ground cytoplasm.

A striking feature in both groups of experimentally treated cells was the formation of intranuclear inclusions in the form of central dense-core complexes. This phenomenon was observed predominantly in B-4 cells treated with 0.1 ppm copper. The inclusions are not a characteristic feature of control cells (Fig. 1 a).

The nuclear inclusions ranging from 0.10 to 0.3 μm in diameter occurred either singly or in small groups (Fig. 1 b). The inclusions consisted of individual or discrete circular, perhaps spherical subunits arranged concentrically or spirally. These subunits appeared evenly distributed in some inclusions whereas in others the core of the inclusion was relatively dense. The number of subunits composing a nuclear inclusion did show variation, and the number of separate subunits in the same nucleus also varied. Variations in density, arrangement, and compactness may be seen. In certain micrographs (Fig. 1 c), it could be seen that each individual constituent subunit was itself a composite structure. The edges of the spherical structures showed several circular densities arranged around an electron-dense center composed of regions of greater and lesser electron density and exhibiting a roughly stellate outline. These inclusions were generally distributed randomly in the nucleoplasm,
FIGURE 1 Transmission electron micrographs of *Scenedesmus* B-4 cells. (a) B-4 control cell, x 53,200. (b) B-4 cell cultured in 0.1 ppm copper, showing distinct nuclear inclusions which exhibit dense cores with scattered electron-dense spheroids, x 200,000. (c) At high magnification, the individual circular subunits of the nuclear inclusions are recognized, x 220,000. (d) The subunits are seen aggregated adjacent to the nuclear membrane and in contact with pores in the membrane (arrows). In the adjacent cytoplasm (Cyt) may be seen subunits of the same relative size and density, x 187,500.
sometimes quite near the nucleolus. They have never been observed in the nucleolar region in copper-treated B-4 cells. In contrast, rounded clumps of electron-dense material identical to the inclusions were recognized in the nucleoli of the copper-treated lab strain Scenedesmus.

Positive identification of the elements contained in the nuclear inclusions was obtained by means of an energy dispersive spectrometer using X-ray energies between 6 and 10 KeV, and revealed peaks at Kα and Kβ lines for copper (Fig. 2). Over 20 areas containing nuclear inclusions were examined and compared to background. The characteristic peaks for copper in this energy area are found at 8.040 (Kα line) and 8.904 (Kβ line), and were consistently observed over the inclusions in unstained sections. No other heavy metals were detected apart from the osmium used for fixation. The large Kβ peak for osmium at 8.910 was inevitable because of the necessity for postfixation to ensure adequate preservation of cellular detail. Copper was not detected in the adjacent cytoplasm. In the absence of other chemical or cytochemical data, however, it is difficult to surmise the exact nature of the nuclear inclusions.

Although the inclusions were always found in the nuclei, they were sometimes found also in the cytoplasm. Of special interest is the extension of the nuclear inclusions into the cytoplasm through pores of the nuclear membrane. That is to say, in some micrographs (Fig. 1 d) the inclusions are continuous with the adjoining cytoplasm through pores of the nuclear membrane. These inclusions often showed extensions in the form of dumbbell-like structures that were directed toward and were sometimes continuous with nuclear membrane pores. Whether inclusions are formed only in the cytoplasm and then move across the nuclear membrane or whether they may form in either site is not yet known.

DISCUSSION

The formation of insoluble, intranuclear metal-protein complexes is characteristic of some forms of heavy metal poisoning, particularly lead, bismuth, and zinc (4). They have been reported in a variety of animal tissues (1, 7, 10), and in moss leaf cells (11). The origin of the protein in the inclusion bodies has been of great interest but has remained speculative (5, 6). It has been suggested (10) that the binding of such metals that occurs in the inclusion body serves as an adaptive or protective mechanism during transcellular transport. This mechanism has the effect of maintaining a relatively low cytoplasmic concentration of the metal and, therefore, reducing the toxic effects of metals on sensitive cellular functions (10). Suggestions concerning the relative metabolic inertness of intracellular metal complexes receive some support in view of the finding that copper inclusions in the nucleus of B-4 cells are found at a concentration of copper lower than that which produces any signs of copper toxicity (14).

The biotic effects of copper pollution are of particular interest because, although copper is an essential trace element for both plants and animals, it is strongly toxic at quite low concentrations. Although the toxicity of copper to different algal types varies greatly (8), the development of copper-tolerant strains has been reported for several algal species (3). The results of the present study which show that copper is incorporated into the nuclei of copper-treated Scenedesmus cells are
the first to indicate such a tolerance mechanism in the algae. Furthermore, the study adds to the growing list of presently known metal pollutants capable of being sequestered in the form of intranuclear inclusions.

It is suggested that the inclusions, described herein, result from a cellular detoxifying mechanism in which copper is complexed by protein ligands, presumably, and that by this mechanism cytoplasmic organelles are to a certain extent protected from the toxic effects of copper. Further characterization of the metal inclusions must await analyses of partially purified intranuclear inclusions isolated by cell fractionation.

SUMMARY
The results of ultrastructural studies and transmission electron microscope microanalysis of two Scenedesmus strains experimentally exposed to copper sulfate are presented. A fine-structural examination of the cells revealed the presence of nuclear inclusions in the form of central dense-core complexes. Cytoplasmic structures resembling the intranuclear inclusions were occasionally found in the cells. TEM-X-ray microanalysis of these structures has provided evidence that the inclusions contain copper. It is concluded that their presence may be regarded as a detoxifying mechanism.

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