Microbial diffraction gratings as optical detectors for heavy metal pollutants

David Noever, Helen Matsos, Andrew Brittain, Don Obenhuber, Raymond Cronise, and Shannon Armstrong

Biophysics Branch, ES-76, NASA Marshall Space Flight Center, Huntsville, Alabama 35812

O24 787

As a significant industrial pollutant, cadmium is implicated as the cause of itai-itai disease. For biological detection of cadmium toxicity, an assay device has been developed using the motile response of the protozoa species, *Tetrahymena pyriformis*. This mobile protozoa measures 50 μm in diameter, swims at 10 body lengths per second, and aggregates into macroscopically visible patterns at high organism concentrations. The assay demonstrates a Cd^{+2} sensitivity better than 1 μM and a toxicity threshold to 5 μM, thus encouraging the study of these microbial cultures as viable pollution detectors. Using two-dimensional diffraction patterns within a *Tetrahymena* culture, the scattered light intensity varies with different organism densities (population counts). The resulting density profile correlates strongly with the toxic effects at very low dosages for cadmium (<5 ppm) and then for poison protection directly (with nickel and copper antagonists competing with cadmium absorption). In particular, copper dosages as low as 0.1–0.5 mM Cu have shown protective antagonism against cadmium, have enhanced density variability for cultures containing 1 mM Cd^{+2}, and therefore have demonstrated the sensitivity of the optical detection system. In this way, such microbial diffraction patterns give a responsive optical measure of biological culture changes and toxicity determination in aqueous samples of heavy metals and industrial pollutants. © *1996 American Institute of Physics.* [S0034-6748(96)05102-9]

I. INTRODUCTION

Heavy metal pollutants represent a major aqueous pollutant not only because of their high toxicity, but also because of their ready accumulation in natural sediments (lakes and rivers). Once soil storage begins, rapid amplification of pollutant concentrations begins to appear in higher food chains.[1–5] For cadmium, the causative agent in itai-itai disease, the rapid formation of protein–cadmium complexes gives very long half-lives for escape from mammalian and human bodies. Once challenged *in vivo*, the organism suffers largely without clinically acceptable treatment because buffering alkali and trace metals complicate excretion via biliary action.[6,7]

The present work targets cadmium poisoning as a pollutant of environmental concern. A novel bioassay[8,9] for rapid screening of cadmium dosages is developed which uses motile cells of *Tetrahymena*, monitors their swimming action, then quantifies chemical toxicity based on scattering intensity for two-dimensional Fourier patterns. These Fourier patterns arise directly from the aggregation and density differences observed in this microbe; the optical diffraction is a variant of scattering from a grating or other barrier of different refractive indices. The principal advantage of this method is its potential for very sensitive detection of otherwise subtle biological changes (swimming behavior). Because of its enhanced cadmium detection, the diffraction technique offers an automated scoring of new antipoisoning agents.[10,11]

Previous work[12,13] has characterized the swimming aggregation behavior of motile *Tetrahymena* cells in response to increasing dosages of cadmium,[8] calcium as protection against cadmium, and other heavy metals such as nickel and copper[9] for antagonizing the biological activity of cadmium

in vivo. While the effects of effluent levels on biological function have distinct structural correlates (such as cell size or shape changes), the function of swimming itself drives the observable behavior in dense cultures. In this way, this systematic toxicological study of cadmium poisoning undertook to examine a statistically predictable mosaic of high and low densities (pattern formation) in spontaneously inhomogeneous *Tetrahymena* cultures. As a measure of cadmium toxicity, the bioassay supplies an accessible monitor linked to a biologically well-characterized organism change.

To quantify behavioral changes as a function of dosage, the formation of effective density differences or "optical gratings" of high and low cell counts give results for image analysis in the rapidly expanding field called statistical crystallography.[8,9,14] The present work furthers this effort by characterizing the optical diffraction quality of the inhomogeneous *Tetrahymena* culture. With success, a simpler biological detector of cadmium activity can potentially automate the screening of protective agents or heavy metal antagonists.

II. MATERIALS AND METHODS

To perform the optical diffraction assay, stock cultures of the protozoa, *Tetrahymena pyriformis* (American Tissue Culture Collection, ATCC), were grown axenically in autoclaved proteose peptone/yeast (PPY) medium, as described elsewhere.[8] To summarize the recipe, a fresh 100 mL growth medium was innoculated with 1 mL of cells harvested from stock at a stationary growth phase. After 2 days, when these cultures passed early logarithmic growth, they were divided into equal 100 mL portions and supplemented with cadmium chloride ($CdC_2 \times 2.5 H_2O$) in a fresh 100 mL of medium.

Culture Density and Temporal Growth

(a)
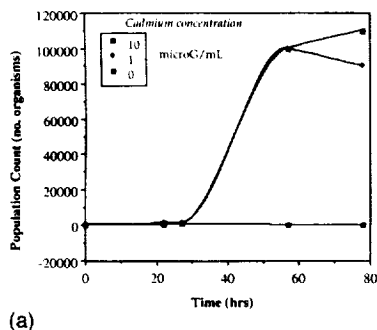
Low Dose Cadmium and Reduced Wavelength

(b)
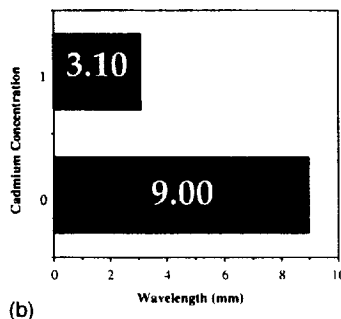
Cadmium Concentration and Delayed Pattern Formation Times
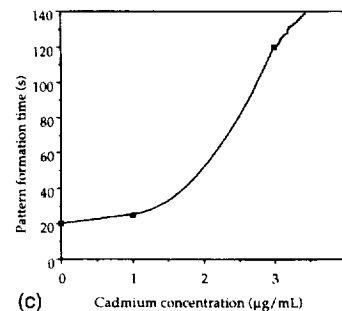

(c)

FIG. 1. The effects of cadmium on the (a) population density, (b) characteristic wavelength of pattern formation, and (c) pattern formation time after manual mixing. The results show a marked effect of cadmium on biologically relevant pattern formation.

Final cadmium concentration varied between 1 and 5 μM. Cultures were incubated at 28 °C constant temperature. The growth of *Tetrahymena* was monitored by microscope (10×) and manual cell counting using a hemacytometer. Solutions were made from 200 μM CdCl$_2$ stock and solutions of nickel and copper buffered stock (in PPY media), seeded with 5 mL *Tetrahymena* stock, then brought up to 200 mL final volume.

Within 2–3 days following a culture reaching its exponential growth phase, bioconvective patterns were induced by first concentrating the cultures. This was accomplished in 200 mL portions, wherein the cells were harvested by hydrostatic filtration through a 0.2 μm mesh. Each culture was filtered to a 10 mL total volume, thus yielding a maximum organism density of approximately 10^6 mL^{-1}. The assay was carried out in Petri dishes, with a culture and media volume measured at 4.8 cm in diameter and 0.8 mm in depth. Multiple 10 mL lots of concentrated cultures (media plus cells) were transferred from filters to dishes using 5 mL plastic pipettes.

Protozoan growth was assessed quantitatively by digitiz-

ing (512×512 pixel resolution) the macroscopic patterns of high and low *Tetrahymena* concentrations, then taking the optical diffraction image (two-dimensional fast Fourier transform). The density difference between high and low organism counts was measured as a factor between 10 and 20. The transformed images were evaluated for peak intensity and angular spread.

III. RESULTS AND DISCUSSION

The cadmium toxicity itself was tested by examining the effects of cadmium concentrations between 1 and 5 μM CdCl$_2$, without protective buffering or antagonism with copper or nickel. Compared to previous tests using mammalian cultures, swimming *Tetrahymena* cells display a remarkably similar correspondence to the lowest observable toxic thresholds (1 μM Cd^{+2}). This similarity lends credence to a comparable biological action of cadmium on both single cells and whole organisms.

Pattern Changes as a Function of Space
and Chemical Concentration (Cd)
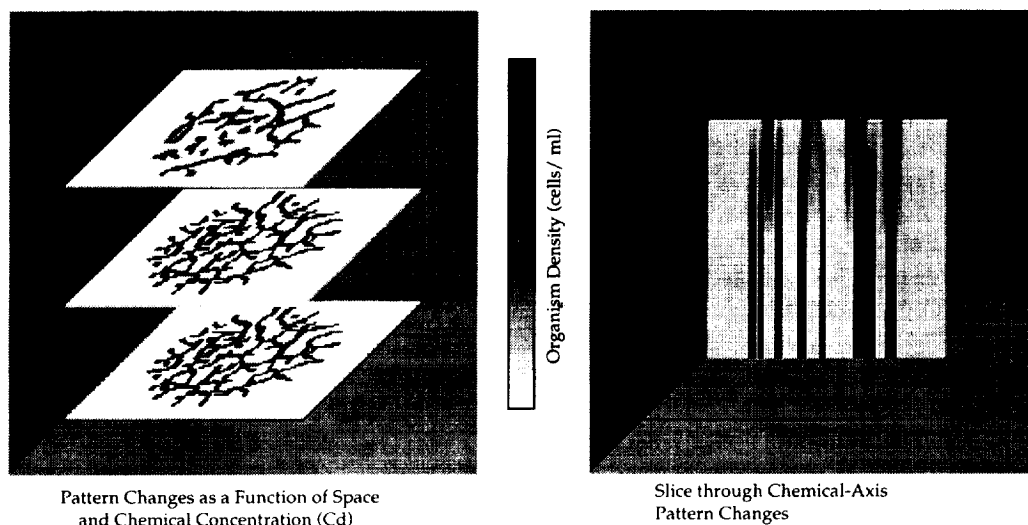
Slice through Chemical-Axis
Pattern Changes

FIG. 2. Effect of cadmium on the growth of *Tetrahymena pyriformis* population. The cultures were supplemented with cadmium. Control cells are grown in PPY media and pattern formation is calibrated to 100% on PPY media without cadmium treated cells. Cd concentrations=1–5 μM. (a) Spatial dimensions represent two axes in a plane and (b) vertically represented cadmium concentration for otherwise identical test cultures.
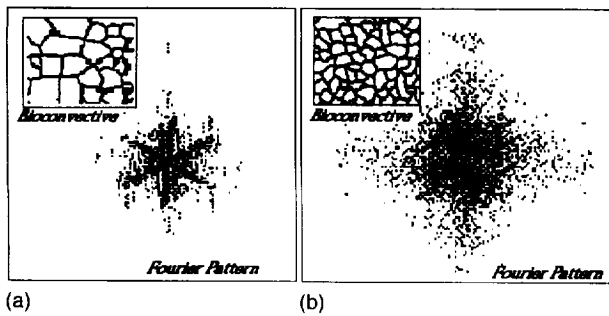
FIG. 3. The effects of time on the real-space and Fourier-transformed patterns of aggregation. (a) shows the pattern 1 h following initial organism concentrations of 10 organism/mL; and (b) shows the same culture 12 h afterward.

The effect of cadmium addition on a culture was seen in its ability to change the observable patterns of organism aggregation. Figure 1 shows three such patterns with increasing cadmium concentrations. Pattern formation disappears entirely above 7 ppm cadmium.

Each cadmium dilution inhibited growth sharply over a narrow range (Fig. 2) and in agreement with previous work.[8,9] The median inhibitory (control) concentration was estimated to be between 5 and 7 μM Cd^{+2}. The concentration-dependent development of cadmium toxicity affects three elements of the detection system: (1) the density of viable organisms surviving [populations count; Fig. 1(a)];

(2) the characteristic wavelength (3–9 mm) of the aggregation pattern in the culture [Fig. 1(b)], and (3) the typical time for an aggregation pattern to form (20–120 s) following stirring or mixing of the sample [Fig. 1(c)]. The latter effect is a direct and macroscopic measure of the robustness of the culture, the swimming speed of its constituent cells, organism count, and overall biological metabolism in the presence of heavy metal pollutants. The generalization of these subtle changes in biological activity are scored presently using optical diffraction from the density differences in such spontaneously inhomogeneous cultures.

The effects of time on a culture's pattern forming ability is illustrated in Fig. 3. In general the effects of time are small if the culture is harvested after its logarithmic growth period (2–3 days following seeding) and monitored over a relatively shorter (2–3 h) observation period. Some cultures have maintained static patterns which change relatively slowly over the course of days to even a week. Figure 3 compares a culture at 1 h after concentration to 10^5 organisms/mL [Fig. 3(a)] to the same culture 12 h afterward [Fig. 3(b)]. The typical wavelength of the pattern has decreased by a factor of 3.2 and the Fourier pattern of optical diffraction has become randomized without the symmetry axes shown in Fig. 3(a). This change furthermore shows the potential for applying the optical diffraction method, since the real space patterns are distinguishable only by changes in
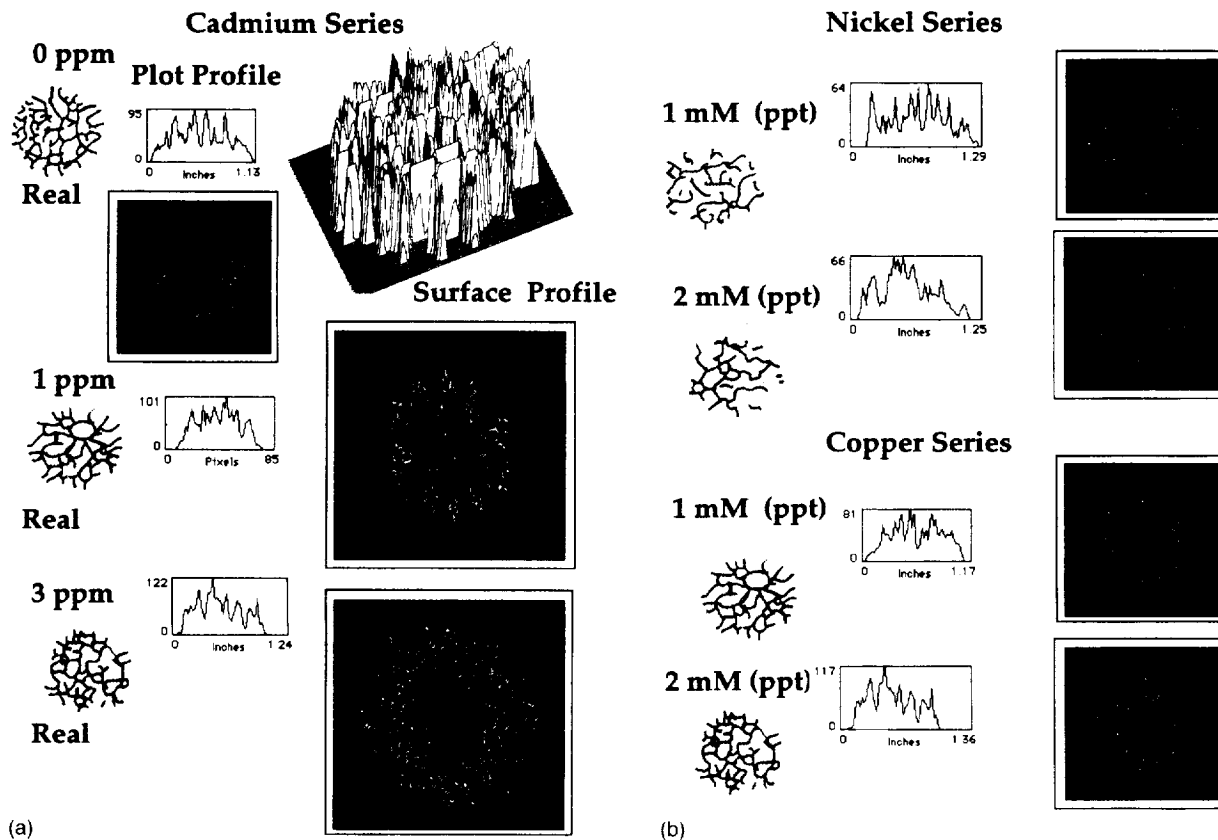

(a)

(b)

FIG. 4. Microbial diffraction gratings shown in real space with random network connections and Fourier transformed counterparts included for the quantification of network changes with cadmium dosages. Bioconvective pattern area properties for *Tetrahymena pyriformis* as (a) a function of Cd concentration between 1 and 3 μM Cd and (b) 1 μM Cd^{+2} for increasing nickel (b) and copper concentrations (c). Plotted are real space planar images of digitized cell concentrations—and corresponding Fast Fourier images of optical diffraction—and scattering intensity as a function of angular position and spot brightness.
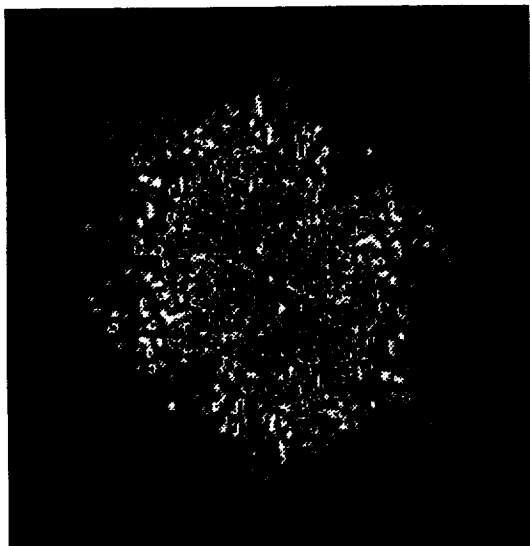
Calculated Fine-Scale Fourier Pattern

(a)
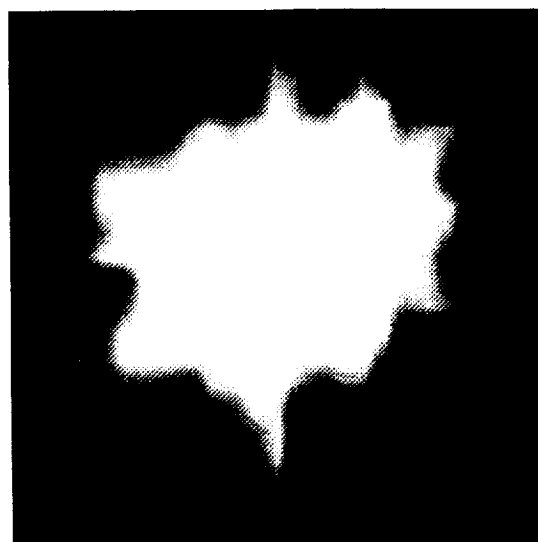
Laser Diffraction (He-Ne) Diffraction Spot (Coarse-Grain)

(b)

FIG. 5. Laser illuminated density pattern from density differences during solution growth of cadmium-doped cultures of *Tetrahymena*. Cadmium concentration equals 1 μM. The He–Ne laser produced 50 mW at 632.8 nm wavelengths. (a) shows the digitized trace from a pattern which is illuminated and (b) (right) shows the direct illumination pattern from the cell culture density scattering itself.

characteristic wavelength, while the Fourier space patterns gives a better measure for distinguishing changes in pattern orientation (e.g., randomized patterns).

The dosage results for the optical diffraction method (Fig. 4) paralleled previous growth studies[1,8,9] but with greater sensitivity in the 1–5 μM Cd^{+2} range. Cadmium concentrations as low as 1 ppm gave a distinct signature on the Fourier transformed images of *Tetrahymena* swimming and aggregation behavior. Since above 5 ppm cadmium inhibits pattern formation altogether, the disappearance of scattered light is easily detectable as the loss of the angular spread in the output signal (due to diffraction from culture density differences shown in Fig. 4). In this way the input signal is attenuated or simply becomes a scintillation detector. The ability to distinguish subtle differences in the Fourier space patterns of Fig. 4(a) is a well-known crystallographic practice, but for the present analysis, it is sufficient to describe simple changes in symmetry properties comparing otherwise identical cultures except for cadmium concentration. Inset to each real and Fourier space pattern is the line profile across each real space pattern. The line profile highlights the inhomogeneity of these spontaneous aggregation patterns and offers the quantitative characterization using, for example, fractal dimension of the line profile or other spectral analyses.

Both the angular distribution of scattering centers and their maximum (peak) intensity differed visibly between the cases of 0, 1, and 3 ppm. Nickel alone showed no consistent effect on patterns at these low dosages and variations shown give a measure of statistical and optical noise [Fig. 4(b)]. Pattern responses for cadmium in the presence of copper

antagonism, however, did show distinct diffraction patterns (0–2 mM Cu) as summarized in Fig. 4(b). The real space pattern of the copper protected cell cultures (2 mM Cu) shows a mean wavelength of 3.3 mm which is distinguishable from the 9 mm wavelength characteristic for cadmium-poisoned samples and consistent with the 3.1 mm average wavelength for undoped cultures.

An alternative means was investigated for generating the Fourier space pattern. Unlike the previous results derived from optically illuminating the digitized trace of pattern inhomogeneities, a He–Ne laser was used to illuminate the cell cultures in real time. The results are shown in Fig. 5(a) for the digitized pattern trace and in Fig. 5(b) for the real-time cell culture. While the fine-scale resolution of the digitized version is not apparent, the real-time diffraction spot does reveal the generally random spatial orientation of the density inhomogeneities.

Quantitative pattern analysis for cadmium in the presence of chelators was undertaken previously using techniques of statistical crystallography.[14] These have been reviewed generally for random patterns and particularly for bioconvective assays.[8,9] For different cadmium levels, comparison of polygonal patterns can be aided by a statistical set characterized by, for example, the size and polygonal shape of two-dimensional patterns. In this way, the mosaic formed by cell swimming and aggregation can become a quantifiable measurement tool for scoring environmental toxicants.

Depending on the bioassay preparation (e.g., antagonizing agent, cadmium concentration, etc.) the favored *Tetrahymena* mosaic pattern should affect the area and polygonal sidedness distribution (Fig. 2). These lopsided distributions

give a measure of disorder and can be appropriately called a crystallographic defect.

The metabolic action of metal binding on cell membranes has received considerable discussion, wherein poison inhibition (by nickel or copper) can arise if metal uptake competes with cadmium or otherwise cannot pass cadmium successfully either from extracellular fluid or directly into cell membranes.[7] The solution of cadmium does not readily pass into the cell. In animal tissues, cadmium becomes bound to proteins soon after incorporation into the cell, so inorganic inhibitors of toxicity such as nickel and copper most probably act extracellularly and prior to membrane penetration.[15] A nuclear study of cadmium's biological action has yet to be undertaken.

The primary result of the optical diffraction system is a highly quantitative means for identifying otherwise closely matched and highly disordered cell networks. Such defect generation and disorder in bioassay networks are observable using an optical diffraction system of scattered intensities from inhomogeneous cell densities and can distinguish effluent concentrations as low as 1 ppm in a biologically well-characterized organism.

By supplying a superior optical diffraction method, the rapid screening of cadmium interaction *in vivo*, along with appropriate design of antagonists, can proceed with improved reliability and efficacy. The dose-response curves for cadmium and buffers confirm results from cell cultures, while providing a novel response and quantitative display for comparing toxicity limits. Future work will automate the optical image process for purposes of speed, cost, and remote access to the environmentally threatening pollutant, cadmium.

[1] W. Krawczynska, N. Pivovarova, and A. Sobota, Acta Protozool. **28**, 245 (1989).

[2] V. Sundararaman and S. K. Gupta, Indian J. Exp. Biol. 1990, **28**, 74 (1990).

[3] C. V. Muino, L. Ferrari, and B. Salibian, Bull. Environ. Contam. Toxicol. **45**, 313 (1990).

[4] E. Piccini, P. Irato, and L. Guidolin, Eur. J. Protistol. **26**, 176 (1990).

[5] Y. P. Ting, F. Lawson, and L. G. Prince, Biotech. Bioeng. **37**, 445 (1991).

[6] M. M. Jones, P. K. Singh, G. R. Gale, L. M. Atkins, and A. B. Smith, Toxicol. Appl. Pharmacol. **95**, 507 (1988).

[7] F. Planas-bohne, M. M. Jones, and P. K. Singh, Cell Biol. Toxicol. **7**, 167 (1991).

[8] D. A. Noever and H. C. Matsos, J. Environ. Sci. Health A **26**, 273 (1991).

[9] D. A. Noever and H. C. Matsos, J. Environ. Sci. Health A **26**, 750 (1991).

[10] J. Silverman and S. Pennisi, J. Toxicol. Cut. Ocular Toxicol. **6**, 33 (1987).

[11] M. J. Wren and D. McCarrol, Environ. Pollution **64**, 87 (1990).

[12] D. L. Hill, *The Biochemistry and Physiology of Tetrahymena* (Academic, New York, 1972).

[13] H. Winet and T. L. Jahn, Biorheology **9**, 87 (1972).

[14] D. Weaire and N. Rivier, Contemp. Phys. **25**, 59 (1984).

[15] W. Rau, F. Planas-Bohne, and D. M. Taylor, Cell Biol. Toxicol. **5**, 91 (1989).