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# Bioassay of Genotoxic Effects of Environmental Particles in a Feeding Ciliate

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The ciliated protozoan, Paramecium, can be used to quantitate cytotoxic and genotoxic effects of ingested complex environmental particles. Cytotoxicity is quantitated by the increased proportion of treated versus control cells which do not retain their capacity for normal cell replication. Genotoxic effects are assessed by the increased fraction of nonviable offspring from treated versus control parent cells after the self-fertilization process of autogamy. Since these cells ingest nonnutrient respirable-sized particles, biological activity of intracellular extraction of dusts and fly ash can be compared before and after extraction with polar and nonpolar solvents. Previous studies indicated that coal fly ash was mutagenic in these eukaryotic cells. Mutagenicity of coal fly ash was not detectable after extraction with a concentration of HCl known to remove nonmatrix trace elements. These results suggested that this ciliate bioassay might be a detector of mineral mutagens. Fine particles of the carcinogenic nickel compounds, α-nickel subsulfide, and β-nickel sulfide were compared for their biological activity in this bioassay. Both nickel compounds were ingested by the ciliates and induced heritable damage in the progeny of the treated parent cells.

## Introduction

Nickel is known to be one of the best documented inorganic carcinogens; the carcinogenicity of nickel compounds in man has been well reviewed (*1-6*). Inhalation of nickel compounds has been implicated in the increased incidence of lung, sinonasal and laryngeal carcinomas among nickel refinery workers (*3, 7-13*). Nickel compounds found to be carcinogenic when administered by inhalation or parenteral routes include nickel subsulfide, crystalline nickel sulfide and nickel carbonyl (*14-21*).

In *P. tetraurelia*, there are two kinds of nuclei, the "germ line" micronucleus and the "somatic" macronucleus. The micronucleus is normally silent (*22*), and any lethal dominant or recessive damage present in the micronucleus will be expressed only when the zygote micronucleus differentiates a new macronucleus for the progeny cell after fertilization. Dominant lethal mutations can be expressed as early as the first cell division after autogamy, while recessive lethals can show phenotypic lag. The presence of dominant wild-type genes from the old macronucleus and their products must be degenerated and/or diluted by successive cell generations prior to expression of recessive traits (*23*). Thus, induction of dominant versus recessive mutations can be monitored by examining the timing of the expression of the damage after autogamy. The fraction of dead or slow growing progeny from treated parents reflects the presence of lethal and detrimental mutations in the parental "germ line" micronuclei (*24-32*).

Cytotoxicity versus genotoxic effects can be easily distinguished and monitored. In general, the bioassay involves treatment of parent cells with the test agent, and if the agent is cytotoxic, the cells will be unable to successfully complete division. Genotoxic effects are assayed by determination of the damage expressed in the self-fertilization offspring of parent cells which have undergone a minimum of seven to ten cell divisions in an agent-free medium to deplete the food supply which induces autogamy. Thus, for detection, induced nuclear damage must be heritable and sufficiently nontoxic to allow successive cell division and successful completion of self-fertilization to result in expression of any induced "germ line" micronuclear damage.

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We have taken advantage of the established mutagenesis assay in *P. tetraurelia* as a prescreen for environmental hazards (*33, 34*) which can be inhaled by man (*35, 36*). Upon inhalation, particles show a size-dependent deposition in the lung where phagocytosis and particle surface interactions occur (*37-39*). The ciliated protozoan, *Paramecium tetraurelia*, engulfs respirable-sized particles (*40*) and undergoes the self-fertilization process of autogamy resulting in homozygosity. Thus induced damage to the parental genome by exposure to test agents is expressed in the next generation of progeny cells (*22, 41*).

In the present study, we have capitalized on the fact that protozoans ingest nonnutritive particles (*40, 42*) of a size respired by man. Intracellular extraction of these airborne particles more closely mimics the phagocytosis seen in macrophages of lungs of mice chronically exposed to fly ash particles (*37*) than does the Ames assay or viral bioassays which require extraction prior to mutagenicity testing. The two finest and most respirable coal fly ash fractions collected from the smoke-stack of a power plant were more mutagenic in the Ames Salmonella assay than two coarser fractions (*43*). The acute toxicity of a single intratracheal instillation of $Ni_3S_2$ particles was 12 times as toxic for fine (VMD = 1.8 μm) versus coarse (13.3 μm) particles (*44*). Thus particle size was found to be a significant factor for biological activity for both coal fly ash and $Ni_3S_2$.

Our previous studies with paramecia indicated that coal fly ash was mutagenic before and after heat treatment. Extraction of particles with aqueous acid, but not with the organic solvent dimethyl sulfoxide, removed detectable mutagenic activity. The positive mutagenicity of the heat-treated fly ash as well as the decreased mutagenic activity with acid extraction found in the protozoan bioassay (*35, 36*), but not in the Ames assay (*45, 46*), suggested the possible increased sensitivity of the protozoan bioassay to mineral mutagens. Therefore, the sensitivity of paramecia to the known metal carcinogen nickel subsulfide ($\alpha$-$Ni_3S_2$) and the crystalline nickel monosulfide ($\beta$-NiS) (*18, 19*) was investigated in the present study.

# Materials and Methods

## Stock and Culture Conditions

Strains of *P. tetraurelia* stock 51 were used. Strain A was derived by crosses of stock 51 with stock 169; strain B was derived by crosses of stock 51 with stock 242. Both derived stocks were kindly supplied by Dr. D. Nyberg and have been described previously by Nyberg (*47*).

The culture medium was inoculated 24 hr before use with *Klebsiella aerogenes* and adjusted to pH 6.8 prior to addition of the ciliates. Cells of known age (*35, 36*) were used as a source of parent cells for the bioassay.

## Preparation and Use of Sized Nickel Compounds

The compounds were kindly prepared by D. McNeill, Battelle Columbus Laboratories. The dusts were prepared by grinding repeatedly in a carbon steel grinding chamber of a Spex Model 8000 mixer mill. Dusts were then suspended in the enthanol settling media and settled in an Andraessen pipet for the required time to remove particles greater than the desired size. The desired fine particles had a volume median diameter (VMD) of 1.82 μm with associated geometric standard deviation of approximately 1.55. The fine particles were gently drawn off, centrifuged to remove excess solvent and then vacuum dried. Particle size and morphology were evaluated by light microscopy. X-ray diffraction studies showed no contaminants or species other than nickel subsulfide before and after size separation by sedimentation (McNeill, personal communication). Nickel monosulfide was purchased from Alpha Products and was ground and sized using the same procedures as those employed for nickel subsulfide. X-ray diffraction analysis demonstrated a mixed crystalline structure of nickel monosulfide with the major phase present as $\beta$-NiS.

Although it was the intent of the authors to also utilize the noncarcinogenic amorphous nickel monosulfide in this investigation, some confusion arose regarding the availability of this compound. Subsequent discussions with Drs. Warner and Timberg (personal communication) of INCO Limited indicated that their attempts to synthesize this compound using previously cited techniques resulted in formation of a product with the sulfur to nickel ratio greater than unity and with a total Ni + S content of less than 100%. They have concluded that X-ray diffraction alone is not sufficient to characterize the product precipitated from a solution of nickel chloride with ammonium sulfide. Thus it appears that results of previous investigations using amorphous nickel sulfide should be interpreted with care and that future studies should provide analysis of both the elemental composition and the crystallinity.

## Cytotoxicity Assay

For biological testing, the particles were suspended in sterile distilled water and sonicated for 15 min to deagglomerate all particles. Suspensions were made 1-2 hr before use to minimize solubility of both nickel monosulfide and nickel subsulfide.

The particles were then diluted in bacterized culture medium before use in the cytotoxicity and genotoxicity bioassays.

Twenty cells were exposed to various dilutions of particles for 2 hr at 34°C and/or 1 day at 27°C. The proportion of viable cells was estimated by the fraction of 20 cells still motile after incubation with the dust suspensions.

## Genotoxicity Assay

The main steps are: (1) treatment of synchronized cells; (2) isolation of single treated cells and growth of each into a clone; (3) induction of autogamy in treated clone; (4) isolation of 16 cells in autogamy, i.e., 16 randomly chosen haploid chromosomal sets from each clone; and (5) determination of how many of these isolates die or grow poorly.

The proportion of autogamous isolates which are nonvigorous or nonviable are presumed to represent an index of the deterimental or lethal mutations in parent cells which are heritable and therefore expressed in their progeny. Self-fertilization results in homozygosity of all genetic loci and therefore expression of induced and/or spontaneous genetic damage.

Synchronized cells were obtained by removing cells with the typical morphology of dividers using a micropipet under a dissecting microscope from a mass culture of exponentially growing cells of known clonal age. The dividers were then incubated at 27°C for 2 hr to provide cells which were at a stage refractory to DNA repair (*29*). These parent cells were then exposed to bacterized culture medium for 2 hr at 34°C, including (1) medium only; (2) sterile washed glass beads, 1-3 μm diameter as a negative control for nonnutritive particles; (3) certain dilutions of the nickel compounds; or (4) the positive control mutagen, 4-nitroquinoline-*N*-oxide. After treatment, the cells were washed by three repeated transfers of cells to agent-free medium with a micropipet under a dissecting microscope. The washed cells were placed as single isolates into fresh media, allowed to multiply and starved to induce the self-fertilization process of autogamy. Samples of cells were stained with acridine orange (*48*), and when 90-100% of the cells showed the nuclear changes typical of autogamy, 16 cells were taken as a source of progeny cells and placed as single isolates into fresh food. Normally 20 parent cells were used for each experimental group to provide 320 progeny (20 × 16). The cells were scored as follows: viable, cells which have cleared the medium of bacteria; detrimentals or slow growers, cells which have not cleared the medium in 3 days; and lethals, no surviving or a few moribund cells. The statistical analysis for determination of significance involved placing confidence intervals on the difference between any two proportions in pairwise comparisons using the normal approximation (*49*).

If the confidence interval did not capture zero, then the pair compared were deemed significantly different. The variance of that difference was computed on the basis of stratified random sample (*50*). The detailed procedure has been described previously (*35, 36*).

Experimental results are presented only for those studies in which the negative control did not differ significantly from mass culture media, since contaminated clones can sometimes yield false positive results.

# Results

Light microscopic observation revealed that both nickel compounds were ingested by paramecia into

**Table 1. Cytotoxicity of nickel dusts.[a]**

| | | Survival, % | | | |
|---|---|---|---|---|---|
| | | Clone A | | Clone B | |
| Time after dose, hr | Dose, μg/mL | NiS | $Ni_3S_2$ | NiS | $Ni_3S_2$ |
| 2 | 0.54 | 100 | 100 | 100 | 100 |
| | 5.4 | 100 | 100 | 100 | 100 |
| | 54 | 100 | 100[b] | 100 | 100[b] |
| | 540 | 100 | 65 | 80 | 35 |
| | 1080 | 65 | 65 | 30 | 25 |
| 24 | 0.54 | 100 | 100 | 100 | 100 |
| | 5.4 | 100 | 100 | 100 | 100 |
| | 54 | 65 | 75 | 100[c] | 90[c] |
| | 540 | 55 | 0 | 15 | 0 |
| | 1080 | 0 | 0 | 0 | 0 |

[a]The percentage of 20 isolated cells still alive after 2 hr and 1 day is given; clones A and B were 20-26 fissions old.
[b]Cells were seen to aggregate around only these nickel particles at this concentration.
[c]Growth inhibition was observed at this concentration.

Table 2. Percent progeny damage after parental exposure to nickel dusts.[a]

| Experimental group | Lethals and detrimentals, % | | | | | |
|---|---|---|---|---|---|---|
| | Expt. 1 | Expt. 2 | Expt. 3 | Expt. 4 | Expt. 5 | Expt. 6 |
| Media only | 1.2 | 0.3 | 12.0 | 0.9 | 2.7 | 5.3 |
| Glass beads | – | 1.1 | 13.8 | 1.3 | 1.3 | 5.6 |
| Nickel subsulfide | | | | | | |
| 0.5 μg/mL | – | – | – | 3.3* | – | 14.2* |
| 5.4 μg/mL | 4.0* | – | – | Died[b] | 3.9‡ | 12.8* |
| 54 μg/mL | 5.5* | 3.4* | 11.9 | – | 1.0 | – |
| ≥270 μg/mL | – | Died | Died[b] | – | – | – |
| Nickel monosulfide | | | | | | |
| 0.5 μg/mL | – | – | – | 5.2* | 1.1 | 10.8* |
| 5.4 μg/mL | – | – | – | 1.6 | 1.6 | 3.3‡ |
| 54 μg/mL | – | 0.6 | 10.8 | – | – | – |
| ≥270 μg/mL | – | 0.8 | 15.9† | – | – | – |
| Clonal age in fissions (strains) | 39(A) | 47(B) | 34(A) | 29(A) | 61(B) | 61(B) |

[a]The absence of values indicates the experimental group was not included.
*Significantly different ($p < 0.01$) from negative controls (media only and glass beads).
†Significantly different from media only, but not glass beads.
‡Significantly different from glass beads, but not from media only.
[b]Died indicates cytotoxicity to parent.

food vacuoles, and these vacuoles appeared swollen relative to those vacuoles which appeared to contain only bacteria.

Cytotoxicity was generally not observed at 54 μg/mL using NiS or $Ni_3S_2$ unless exposed overnight (Table 1). However, older clones (61 fissions old) of strain B died at this concentration and at a 10-fold dilution of nickel subsulfide after a 2 hr exposure. The age or differences in strain sensitivity to nickel compounds may contribute to the observed cytotoxicity since strain B was more sensitive than strain A after 2 hr exposures to the nickel compounds (Tables 1 and 2).

In four of six experiments, $Ni_3S_2$ showed significantly ($p < 0.01$) higher lethality and detrimentals compared to the media only or negative glass beads controls (Table 2). In the one negative experiment, the baseline mutagenesis was high (12%), and the most genotoxic concentration (0.5 μg/mL) was not tested. Nickel monosulfide yielded progeny with increased lethality and detrimentals relative to controls in three of five experiments. Both nickel compounds show a decrease in biological effect with increase in concentration (Table 3).

Table 3. Mean percent lethality and detrimentals after parental exposure to nickel dusts.[a]

| Experimental group | Lethals and detrimentals, % | Total progeny |
|---|---|---|
| Media only | 2.1 | 3104 |
| Glass beads | 2.3 | 2560 |
| Nickel subsulfide | | |
| 0.5 μg/mL | 8.8* | 1280 |
| 5.4 μg/mL | 6.9* | 1696 |
| 54 μg/mL | 3.3 | 1584 |
| Nickel monosulfide | | |
| 0.5 μg/mL | 5.2* | 1744 |
| 5.4 μg/mL | 1.8 | 2480 |
| 54 μg/mL | 0.8 | 608 |
| 4-Nitroquinoline-*N*-oxide 1.9 μg/mL[b] | 17.5 | 2448 |

[a]The mean values represent the pooled individual experiment from Table 2 with the exception of experiment 3. The exclusion was made because this clone contained high baseline mutagenesis not seen in the other series.
[b]The positive control used was the direct-acting mutagen, 4-nitroquinoline-*N*-oxide, derived from cumulative experiments. In some experiments, the carcinogen was toxic to the parent cells used and no value could be obtained.
*These values are significantly different ($p < 0.01$) from the negative control glass beads and media only.

## Discussion

Both the nickel subsulfide and the crystalline nickel monosulfide dusts significantly increased lethality among the self-fertilization offspring from treated versus control parents. The monosulfide exhibited more variability and less severity than the subsulfide with respect to loss of progeny viability and vigor. No difference in the ability of the paramecia to ingest the two nickel dusts was observed, although quantitative studies were not carried out.

The decrease in genotoxicity with increased dose of nickel dusts was not due solely to elimination from the population of damaged cells by death since lethality and detrimentals are derived from offspring of viable parents. Some damaged parent cells could be eliminated from the experimental pool by cytotoxicity.

The inverse dose-response relationship may reflect nickel-induced changes in the physiology of the cell which promotes protection of the cell from heri-

table damage. Any reduction in the rate of uptake and/or reduction in growth rate could decrease damage and promote repair before DNA replication.

The Paramecium bioassay did not show a striking difference in the biological activity of the $\alpha$-$Ni_3S_2$ and $\beta$-NiS, in agreement with Sunderman and Hopfer (*14*), who indicated that both are metal carcinogens in rats. Similarly Costa et al. (*51*) reported that crystalline nickel subsulfide and nickel monosulfide described herein also produce neoplastic transformations in Syrian hamster embryo cultures.

There is also a correlation between induction of heritable damage in paramecia and nickel-compound induced damage in plant cells. Abnormalities have been observed in root cells of the broad bean induced by nickel compounds (*52-54*).

In summary, with respect to the carcinogenicity of these two nickel compounds in experimental animals, this bioassay demonstrated a correlation between the eukaryotic cell mutagenicity and previously published reports on the animal carcinogenicity or the two agents.

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## REFERENCES

1. Kasprzak, K. S., and Marchow, L. The carcinogenic action of nickel subsulfide, $Ni_3S_2$. Patol. Polska 23: 135-142 (1972).
2. Sunderman, F. W., Jr., Coulston, F., Eichhorn, G. L., Fellows, J. W., Mastromatteo, E., Reno, J., Samitz, M. H., Curtis, B. L., Vallee, B. L., West, P. W., McEwan, J. C., Shibco, S. J., and Boaz, J. D. (Eds.), Committee on Biological Effects of Atmospheric Pollutants, Nickel. National Academy of Sciences, Washington, DC, 1975, p. 144.
3. Sunderman, F. W., Jr. Nickel carcinogenesis. Disease Chest 54: 41-47 (1968).
4. Sunderman, F. W., Jr. Metal Carcinogenesis. In: Advances in Modern Toxicology, Vol. 2 (R. A. Goyer and M. A. Mehlman, Eds.), Hemisphere Publ. Corp., Washington, DC, 1977 pp. 257-282.
5. Sunderman, F. W., Jr. Recent research on nickel carcinogenesis. Environ. Health Perspect. 40: 131-141 (1981).
6. Raithel, H. J., and Schaller, K. H. Toxicity and carcinogenicity of nickel and its compounds. A review of the current status. Zentralbl. Bakteriol. B. 173: 63-94 (1981).
7. Morgan, J. G. Some observations on the incidence of respiratory cancer in nickel workers. Brit. J. Ind. Med. 15: 224-234 (1958).
8. Doll, R. Cancer of the lung and nose in nickel workers. Brit. J. Ind. Med. 15: 217-223 (1958).
9. Mastromatteo, E. Nickel: a review of its occupational aspect. J. Occup. Med. 9: 123-136 (1967).
10. Doll, R., Morgan, L. G., and Speizer, F. E. Cancer of the lung and nasal sinuses in nickel workers. Brit. J. Cancer. 24: 623-632 (1970).
11. Doll, R., Mathews, J. D., and Morgan, L. G. Cancers of the lung and nasal sinuses in nickel workers: a reassessment of the period of risk. Brit. J. Ind. Med. 34: 102-105 (1977).
12. Kreyberg, L. Lung cancer in workers in a nickel refinery. Brit. J. Ind. Med. 35: 109-116 (1978).
13. Lessard, R., Reed, D., Maheus, B., and Lambert, J. Lung cancer in New Caledonia: A nickel smelting island. J. Occup. Med. 20: 815-817 (1978).
14. Sunderman, F. W., Jr., and Hopfer, S. M. Correlation between the carcinogenic activities of nickel compounds and their potencies for stimulating erythropoiesis in rats. In: Biological Aspects of Metals and Metal-Related Diseases (B. Sarkar, Ed.), Raven Press, New York, 1982.
15. Lau, T. J., Hackett, R. L., and Sunderman, F. W., Jr. The carcinogenicity of intravenous nickel carbonyl in rats. Cancer Res. 32: 2253-2258 (1972).
16. Ottolenghi, A. D., Haseman, J. K., Payne, W. W., Salk, H. L. and MacFarland, H. M. Inhalation studies of nickel sulfide in pulmonary carcinogenesis of rats. J. Natl. Cancer Inst. 54: 1165-1172 (1977).
17. Huberman, E., Mager, R., and Sachs, L. Mutagenesis and transformation of normal cells by chemical carcinogens. Nature 264: 360-361 (1976).
18. Sunderman, F. W., Jr. and Maenza, R. M. Comparisons of carcinogenicities of nickel compounds on rats. Res. Comm. Chem. Pathol. Pharmacol. 14: 319-330 (1976).
19. Sunderman, F. W., Jr., Maenza, R. M., Allpass, P. R., Mitchell, J. M., Damjanov, I., and Goldblatt, P. J. Carcinogenicity of nickel subsulfide in Fischer rats and Syrian hamsters after administration by various routes. In: Inorganic and Nutritional Aspects of Cancer (G. N. Schrauzer, Ed.), Plenum Press, New York, 1978, p. 67.
20. Yarita, T., and Nettesheim, P. Carcinogenicity of nickel subsulfide for respiratory tract mucosei. Cancer Res. 38: 3140-3145 (1978).
21. Damjanov, I., Sunderman, F. W., Jr. Mitchell, J. M., Allpass, R. P. Induction of testicular sarcoma in Fischer rats by intratesticular injection of nickel subsulfide. Cancer Res. 38: 268-276 (1978).
22. Sonneborn, T. M. Methods in Paramecium research. In: Methods in Cell Physiology (D. M. Prescott, Ed.), Academic Press, New York, 1970, pp. 241-251.
23. Berger, J. Gene expression and phenotypic change in *Paramecium aurelia* exconjugants. Genet. Res. 27: 123-124 (1976).
24. Kimball, R. F. The affect of radiation on genetic mechanisms of *Paramecium aurelia*. J. Cell Comp. Physiol. 35: 157-169 (1950).
25. Kimball, R. F. X-ray dose rate and dose fractionation studies on mutations in *Paramecium*. Genetics 48: 581-595 (1963).
26. Kimball, R. F. The induction of repairable premutational damage in *Paramecium aurelia* by the alkylating agent triethylene melamine. Mutat. Res. 2: 414-425 (1965).
27. Kimball, R. F. Studies on mutations induced by ultraviolet irradiation in *Parmaceium aurelia* with special emphasis on photoreversal. Mutat. Res. 8: 79-89 (1969).
28. Kimball, R. F. and Gaither, N. The influence of light upon the action of UV on *Paramecium aurelia*. J. Cell Comp. Physiol. 37: 211-233 (1951).
29. Kimball, R. F., and Perdue, S. W. Studies on the refractory period for the induction of recessive lethal mutations by X-rays in Paramecium. Genetics 49: 1595-1607 (1962).
30. Kimball, R. F., and Perdue, S. W. Comparison of mutagenicity of X-rays and triethylene melamine in Paramecium with emphasis on the role of mitosis. Mutat. Res. 4: 37-50 (1967).
31. Powers, E. L., Jr. Radiation effects in Paramecium. Ann. NY Acad. Sci. 57: 619-630 (1955).
32. Rodermel, S., and Smith-Sonneborn, J. Age-correlated changes in expression of micronuclear damage and repair

in *Paramecium tetraurelia.* Genetics 87: 259-274 (1977).
33. Smith-Sonneborn, J. DNA repair and longevity assurance in *Paramecium tetraurelia.* Science 203: 1115-1117 (1979).
34. Smith-Sonneborn, J. Use of a ciliated protozoan as a model system to detect toxic and carcinogenic agents. In: In-Vitro Toxicity Testing of Environmental Agents: Current and Future Possibilities. Monte Carlo Proc. NATO Adv. Res. Inst. Part A. A survey of test systems. (A. R. Kolder, T. K. Wong, L. D. Grant, R. S. DeWoskin, and T. J. Hughes, Eds.), Plenum Press, New York, 1982, pp. 113-138.
35. Smith-Sonneborn, J. Palizzi, R. A., Herr, C., and Fisher, G. L. Mutagenicity of fly ash particles in Paramecium. Science 211: 180-182 (1981).
36. Smith-Sonneborn, J. Fisher, G. L., Palizzi, R. A. and Herr, C. Mutagenicity of coal fly ash: A new bioassay for mutagenic potential in a particle feeding ciliate. Environ. Mut. 3: 239-252 (1981).
37. Fisher, G. L. and Wilson, F. D. The effects of coal fly ash and silica inhalation on macrophage function and progenitors. J. Reticuloendothel. Soc. 27: 513-527 (1980).
38. Fisher, G. L., and Natusch, D.F.S. Size dependence on the physical and chemical properties of fly ash. In: Analytical Methods for Coal and Coal Products. Vol. 3 (C. Karr, Ed.), Academic Press, New York, 1979, pp. 489-541.
39. Yeh, H. C., Phalen, R., and Raabe, D. B. Factors influencing the decomposition of inhaled particles. Environ. Health Perspect. 15: 147-156 (1976).
40. Smith-Sonneborn, J., and Rodermel, S. R. Loss of endocytic capacity in aging Paramecium: the importance of cytoplasmic organelles. J. Cell Biol. 71: 575-588 (1976).
41. Sonneborn, T. M. *Paramecium aurelia.* In: Handbook of Genetics, Vol. 2 (R. C. King, Ed.), Plenum Press, New York, 1974, pp. 469-594.
42. Fenchel, T. Suspension feeding in ciliated protozoa: functional response and particle size selection. Micro. Ecol. 6: 1-11 (1980).
43. Fisher, G. L., Chrisp, C. E., and Raabe, O. G. Physical factors affecting the mutagenicity of fly ash from a coal-fired power plant. Science 205: 370-381 (1979).
44. McNeill, D. A., Chrisp, C. E., and Fisher, G. L. Factors affecting nickel subsulfide toxicity. Toxicology 2: 84 (1982).
45. Fisher, G. L., Chrisp, C. E., and Wilson, F. D. Coal fly ash as a model complex mixture for short term bioassay. In.: Second Symposium on Application of Short Term Bioassay in the Fractionation and Analysis of Complex Envimental Mixtures (M. D. Waters, S. S. Sandhu, J. L. Huisingh, L. Claxton and S. Nesnow, Eds.) Williamsburg, VA 1980.
46. Chrisp, C. E., and Fisher, G. L. Mutagenicity of airborne particles. Mut. Res. 76: 143-164 (1980).
47. Nyberg, D. J. Genetic analysis of trichocyst discharge of the wild stocks of *Paramecium tetraurelia.* Protozool. 25: 107-112 (1978).
48. Smith-Sonneborn, J. Acridine orange fluorescence: A temporary stain for paramecia. Stain Technol. 49: 77-80 (1974).
49. Mendenhall, W. Introduction to Probability and Statistics. Duxbury Press, Belmont, 1975, p. 186.
50. Cochran, W. C. Sampling Techniques. Wiley, New York, 1977, p. 106.
51. Costa, M., Simons-Hansen, J., Bedrosslan, C. W. M., Bonura, J., and Capridl, R. M. Phagocytosis, cellular distribution, and carcinogenic activity of particulate nickel compounds in tissue culture. Cancer Res. 41: 2868-2876 (1981).
52. Glass, E., Untersuchungen uber der Einwirkung von Schwermettalsalzen auf der Wurzelspitzenmitose von *Vicia faba.* Z. Botanik. 43: 359-403 (1955).
53. Glass, E. Verteileng von Fragmentation und achromatischen Stellen aut den Chromosomen von *Vicia faba* nach Behandlung mit Schwermetallsalzen. Chromosoma 8: 260-284 (1956).
54. Komczynski, L., Nowak, H., and Rejniak, L. Effect of cobalt, nickel and iron on mitosis in the roots of the broad bean (*Vicia faba*). Nature 198: 1016-1017 (1963).