Mutagenicity of Filtrates from Respirable Coal Fly Ash

Abstract. *Incubation of histidine-requiring auxotrophs of the bacterium* Salmonella typhimurium *with cyclohexane-, saline-, and serum-soluble surface components of respirable fly ash particles produced an increased number of revertants in two frameshift tester strains. The results are consistent with the hypothesis that both organic and inorganic mutagens are present in coal fly ash.*

Coal combustion for electric power generation is predicted to increase dramatically throughout the remainder of this century. It can be estimated that in 1974 a total of 2.4×10^6 metric tons of fly ash was released to the atmosphere from all coal-burning facilities in the United States (*1*). As part of our studies of the potential health impacts of electrical energy-producing coal combustion technologies, we evaluated the mutagenicity of soluble components of respirable coal fly ash. Recent studies have shown a high positive correlation between carcinogenicity of substances for animals or man and mutagenic activity in a bacterial test system (*2*).

Kilogram quantities of size-fractionated fly ash were collected downstream of the electrostatic precipitator from the smokestack breeching of a large modern power plant burning pulverized low sulfur, high ash coal (*3*). Of the four sized fractions obtained, the finest fraction, with a mass median diameter (MMD) of 2.2 μm and geometric standard deviation (σ_g) of 1.8, is the most biologically important since particles of this size have the longest atmospheric residence time, are most efficiently deposited in deep lung, and are least efficiently removed (*4*). Five strains of histidine-requiring (his^-) auxotrophs of the bacterium *Salmonella typhimurium* (supplied by B. N. Ames) were used. The testing methods and mutations involved have been described (*2*). Briefly, strains TA100 and TA1535 have been used to detect base-pair substitution mutagens; TA1537, TA1538, and TA98 are strains that are susceptible to frameshift mutagens. Strains TA98 and TA100 have an ampicillin-resistant R factor not present in the other strains. Sufficient histidine was added to agar plates to allow the histidine-requiring bacteria to replicate several times in the presence of the test substance. After incubation, the histidine-synthesizing (his^+ revertant) bacteria colonies were counted.

Two media were selected for mutagenicity studies with fly ash. Dulbecco's phosphate-buffered saline was used because it has the *p*H and tonicity of physiological fluids, and horse serum was used because serum has a chemical constituency similar to lung alveolar fluid and forms soluble complexes with some carcinogenic heavy metals (*5*). Fly ash samples were incubated separately with each of these media for 2 weeks at 37°C. After incubation, the fly ash mixtures were centrifuged at 35,000*g*, and the supernatants were passed through a 0.45-μm membrane filter to remove particulate matter. Media controls of serum or saline were treated in the same fashion as the fly ash mixtures. Filtrates were added to filter paper disks in the standard spot test and also added to soft, top agar pour plates. No mutagenic activity was found with the spot test, but revertants were seen with the plate technique. This was evidence that the mutagen or mutagens did not diffuse into the media from the paper disks. Of the five strains tested, TA98 and TA1538 showed his^+ revertants, whereas TA1535, TA1537, and TA100 did not. Because strain TA1538 showed two to three times the number of revertants as TA98, TA1538 was used in subsequent tests with varying concentrations of fly ash. Serum filtrate had approximately tenfold greater activity than the saline filtrate (Fig. 1). Solubility of substances responsible for mutagenic activity in saline, a polar solvent, suggested the presence of polar organic or inorganic mutagens. Although these tests confirmed the presence of direct mutagens, many mutagens require metabolic conversion by cellular microsomal enzymes to active mutagens (*2*). In further experiments, addition of optimal concentrations of rat liver homogenates from rats treated with polychlorinated biphenyl (Arochlor 1254) (*2*) did not significantly increase the number of revertants in either the saline or serum filtrate (Table 1).

Further work was designed to evaluate the chemical nature of the active fly ash components. Fly ash was extracted directly with cyclohexane, a nonpolar, nonmutagenic organic solvent. The extract was evaporated to dryness and reconstituted with dimethyl sulfoxide, and this mixture was tested for mutagenicity. About 40 percent of the mutagenic activity of the serum filtrate was present, and metabolic activation increased the number of revertants 2.2-fold (Table 1). At least a portion of the activity thus can probably be attributed to mutagenic nonpolar organic compounds. In contrast, after extraction of the saline filtrate with cyclohexane, mutagenic activity was found only in the aqueous fraction. This confirmed the polar nature of the activity of the saline-soluble fraction and suggested the presence of at least two mutagenic compounds.

To further assess the chemical speciation of the mutagenic activity in the serum filtrate, 2 m*M* disodium ethylenediaminetetraacetic acid (EDTA), a metal chelator, was added to serum filtrate. The number of his^+ revertants was increased by about 60 percent compared to the untreated filtrate (Table 2). In order to test the hypothesis that EDTA had chelated mutagenic metals complexed with serum proteins, EDTA-treated and untreated serum filtrates were fractionated on a Sephadex PD-10 column with a cutoff at 25,000 daltons. EDTA (2 m*M*) was added to one portion of serum filtrate and stirred overnight at 4°C before elution on the column. A second portion was prepared in the same manner without prior treatment with EDTA. Each of these two filtrates was eluted with three void volumes of double-distilled water. The first fraction contained more than 95 percent of the total serum protein. The second had the remaining protein and a

Table 1. Number of TA1538 His^+ revertants per plate with and without metabolic activation. The concentration of fly ash incubated with serum and saline before filtration was 78 mg/ml; the cyclohexane extract was evaporated under nitrogen and reconstituted with dimethyl sulfoxide to the equivalent of 78 mg/ml. All controls were treated in a manner analogous to the test materials. Spot tests were positive with 4-nitro-quinoline-*N*-oxide without S-9 (the supernatant fraction of rat liver homogenate, centrifuged at 9000*g*) and with 2-aminofluorene and S-9 added. Mean values ± the standard error of the mean (S.E.M.) were for three replicate determinations. The number of spontaneous revertants per plate was 7 ± 1. The number of revertants with addition of S-9 alone was 20 ± 1.

Test media	S-9 not added		S-9 added	
	Fly ash	Control	Fly ash	Control
Serum filtrate*	154 ± 32	10 ± 2	202 ± 18	12 ± 5
Saline filtrate*	17 ± 3	4 ± 1	40 ± 9	16 ± 2
Cyclohexane extract	62 ± 2	5 ± 2	151 ± 8	27 ± 5

*The mutagenic activity of serum and saline filtrates in these studies was somewhat less than that observed in the earlier dose-response experiments (Fig. 1).

0036-8075/78/0106-0073$01.00/0

small amount of low-molecular-weight compounds, while the third fraction contained only low-molecular-weight components. Each of the three fractions was lyophilized and reconstituted with double-distilled water before testing. Regardless of prior treatment with EDTA, the total mutagenic activity in the fractions was lower than that in the original filtrate (Table 2). Of the total net activity after subtraction of background revertants (5.0 ± 1.0 percent), 79, 18, and 3 percent were present in the first, second, and third untreated fractions, respectively. Of the total net activity after subtraction of appropriate control values (Table 2) 83, 0, and 17 percent were found in the three EDTA-treated fractions, respectively. The significant increase ($P < .001$) in the activity of the low-molecular-weight fraction of the EDTA-treated serum filtrate lends credence to the hypothesis that EDTA acted by chelating mutagenic metals from serum proteins. The mutagenic activity was predominantly associated with the fraction of higher molecular weight, with or without EDTA treatment. This activity may be due to organic compounds postulated, as a result of the cyclohexane extraction studies, to be present on fly ash surfaces. Mutagenic organic compounds—for example, polynuclear aromatic hydrocarbons—have been shown to bind to proteins (*6*). Additional explanations for the relative enhancement of the mutagenicity of the serum filtrates compared to the saline filtrates include the possible presence of (i) enzymes in serum capable of converting promutagens to mutagens or (ii) protein-mutagen complexes that are more available to the bacterial cells.

We have studied the trace element composition of the fractionated fly ash. The respirable fly ash fraction, relative to the other sized fractions, has the highest concentration of many elements (*7*) that in some chemical forms have been reported to be mutagenic (*8–13*) or carcinogenic (*14*). In order of decreasing concentration enhancement, relative to the coarsest fraction (MMD = 20 μm; σ_g = 1.9), the following elemental concentrations (micrograms per gram) for some mutagenic or carcinogenic metals analyzed by instrumental neutron activation analysis or atomic absorption spectrophotometry (*15*) in the finest fraction have been determined: Cd(4.6), Se(198), As(132), Sb(20.6), Mo(50), Pb(278), Co(21), Cu(137), Be(10.3), Ni(40), Mn(309), and Fe(32,000). The inverse dependence of concentration on particle size has been explained, for the most part, as being due to condensation of volatile metals and their oxides on the surface of fly ash particles (*16*). Therefore, although average concentrations of potentially toxic materials on the surface of these aluminosilicate spheres (*17*) may be on the order of tens or hundreds of micrograms per gram, surface concentrations may be as high as 1 to 5 percent (*18*). Of the metals found in fly ash, a number have been demonstrated to be mutagenic in the *Salmonella* reversion assay (*8*). These include compounds of chromium (*10, 11*), iron (*12*), manganese (*13*), and selenium (*10*). Sirover and Loeb have suggested that the mechanism of damage by many mutagenic and carcinogenic metals is due to decreased fidelity of DNA synthesis (*19*). Sodium bisulfite, which may be present on the surface of fly ash (*20*), has also been shown to be mutagenic (*21*).

Table 2. Effect of EDTA treatment and serum fractionation on the number of TA1538 His$^+$ revertants per plate. The concentrations of fly ash incubated with serum was 78 mg/ml (as in Table 1). The mean values ± S.E.M. were for five determinations. The number of spontaneous revertants per plate was 5 ± 1. Controls were serum samples with EDTA added and treated in a manner analogous to the test materials. Abbreviation: UF, unfractionated.

Serum filtrate fraction	Fly ash	Fly ash + EDTA	Control
UF	162 ± 18	261 ± 25	8 ± 2
Fraction 1	79 ± 11	94 ± 10	7 ± 1
Fraction 2	21 ± 4	11 ± 4	11 ± 2
Fraction 3	7 ± 2	22 ± 3	4 ± 1

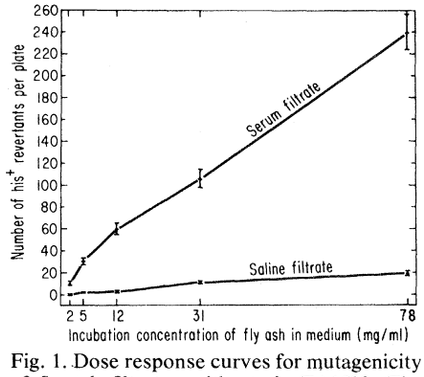

Fig. 1. Dose response curves for mutagenicity of fly ash filtrates with strain TA1538. The number of his$^+$ revertants per plate is the mean of 5 to 20 replicate determinations minus the mean of the appropriate background revertants (serum or saline). The background reversion was defined as the group mean of the spontaneous revertants and the appropriate media control after it was determined that the number of his$^+$ revertants in all negative controls was not significantly different from that of spontaneous revertants. The means (± S.E.M.) of the background revertants were 5.8 (±0.4), 6.9 (±0.9), 4.0 (±0.6) for the spontaneous revertants, serum controls, and saline controls, respectively. Filtrate (100 μl) was added to 2 ml of soft top agar before plating. Plates were incubated for 2 days at 37°C. The vertical bars are 1 S.E.M.

Although we have not analyzed for organic compounds, they (particularly polynuclear aromatic hydrocarbons) have been reported to be present on the surface of fly ash (*22*). Studies of suspended particulates in urban aerosols have also resulted in the identification of polynuclear aromatic hydrocarbons, as well as oxidized species, including polycyclic quinones (*23*). Although most polynuclear aromatic hydrocarbons require metabolic activation, some of the oxides do not (*24*). It has been postulated (*25*) that, in addition to polynuclear aromatic hydrocarbons, urban aerosols contain other extractable organic components that are mutagenic in the Ames test system. Since our fly ash samples were collected at 100°C from the power plant smokestack, concentrations of organic compounds were probably lower than would be present if the fly ash were cooled rapidly to ambient temperatures as occurs in the smokestack plume; however, the organic compounds may be altered by interaction with effluent gases or light and other environmental constituents (*22*).

In summary, evidence has been presented that filtrates from a respirable fraction of coal fly ash collected from a power plant over a 30-day period contain substances that cause frameshift mutations in a bacterial strain lacking normal excision repair. These substances apparently include both organic and inorganic compounds. Increased mutagenicity of the serum filtrate compared to the other filtrates may indicate that extraction with serum increases the sensitivity of the Ames technique for detecting mutagenicity of complex mixtures. It may be expected that substances on the surface of fly ash deposited in the deep lung should be similarly soluble in alveolar fluid. The prospect of a large increase in the amount of coal burned for energy production warrants specific identification of these mutagenic substances and a careful assessment of the possible carcinogenic properties of respirable fly ash.

C. E. CHRISP
G. L. FISHER, J. E. LAMMERT
Radiobiology Laboratory,
University of California, Davis 95616

References and Notes

1. The estimate is based on the ERDA Balanced Program Plan [*Coal Extraction Processing and Combustion* (ERDA Publ. 116, Argonne National Laboratory, Argonne, Ill., 1976), vol. 3, pp. 3 and 16], which indicated that U.S. power plants burned a total of 600 × 10^6 tons of coal of which 11 percent is ash. Eighty percent of the total ash is fly ash of which approximately 5 percent is released to the atmosphere.

2. B. N. Ames, J. McCann, E. Yamasaki, *Mutat. Res.* **31**, 347 (1975).
3. A. R. McFarland, G. L. Fisher, B. A. Prentice, R. W. Bertch, *Environ. Sci. Technol.* **11**, 781 (1977).
4. T. T. Mercer, *Aerosol Technology in Hazard Evaluation* (Academic Press, New York, 1973), pp. 21–62; H. C. Yeh, R. F. Phalen, O. G. Raabe, *Environ. Health Perspect.* **15**, 147 (1976).
5. J. C. Heath, M. Webb, M. Caffrey, *Br. J. Cancer* **23**, 153 (1969).
6. C. Heidelburger, *Annu. Rev. Biochem.* **44**, 79 (1975).
7. G. L. Fisher *et al.*, Second Joint Chemical Institute of Canadian/American Chemical Society Conference, Montreal, 29 May to 2 June 1977.
8. C. P. Flessel, in *Inorganic and Nutritional Aspects of Cancer*, G. N. Schrauser, Ed. (Plenum, New York, in press).
9. T. Rossman, M. S. Meyn, W. Troll, *Mutat. Res.* **30**, 157 (1975); H. Nishioka, *ibid.* **31**, 185 (1975); M. Green, W. Muriel, B. Bridges, *ibid.* **38**, 33 (1976); L. L. Weed, *J. Bacteriol.* **85**, 1033 (1963).
10. G. Löfroth and B. N. Ames, in *Abstracts Environmental Mutagen Society* (Environmental Mutagen Society, Colorado Springs, Colo., February 1977), p. 30.
11. K. R. Tindall, G. R. Warren, P. D. Skaar, in *ibid.*, p. 49.
12. D. Brusick, F. Gletten, D. Jagunnath, U. Weeks, *Mutat. Res.* **38**, 386 (1976).
13. C. P. Flessel, personal communication; W. Prazmo, E. Balbin, H. Baranowska, A. Ejchart, A. Putrament, *Genet. Res.* **26**, 21 (1975).
14. A. Furst, *Proceedings of the International Association of Bioinorganic Scientists*, in press; International Agency for Research on Cancer monographs: *Evaluation of the Carcinogenic Risk of Chemicals to Man* (1972), vol. 1, and *Some Inorganic and Organometallic Compounds* (1973), vol. 2; J. C. Heath, *Br. Emp. Cancer Campaign Rep.* (1963), part 2, p. 389; G. D. Stoner, M. B. Shimkin, M. C. Troxell, T. L. Thompson, L. S. Terry, *Cancer Res.* **36**, 1744 (1976); A. D. Ottolenghi, J. K. Haseman, W. W. Payne, H. L. Falk, H. N. MacFarland, *J. Natl. Cancer Inst.* **54**, 1165 (1975); T. J. Lau, R. L. Hackett, F. W. Sunderman, Jr., *Cancer Res.* **32**, 2253 (1972); A. Furst, M. Schlauder, D. P. Sasmore, *ibid.* **36**, 1779 (1976).
15. J. M. Ondov, R. L. Ragaini, R. E. Heft, G. L. Fisher, D. Silberman, B. A. Prentice, *Proceedings of the Eighth Materials Research Symposium, Methods and Standards for Environmental Measurement* (National Bureau of Standards, Gaithersburg, Md., in press).
16. R. L. Davison, D. F. S. Natusch, J. R. Wallace, C. A. Evans, Jr., *Environ. Sci. Technol.* **8**, 1107 (1974).
17. G. L. Fisher, D. P. Y. Chang, M. Brummer, *Science* **192**, 533 (1976).
18. R. W. Linton, A. Loh, D. F. S. Natusch, C. A. Evans, Jr., P. Williams, *ibid* **191**, 852 (1976).
19. M. A. Sirover and L. A. Loeb, *ibid* **194**, 1434 (1976).
20. L. D. Hansen, D. J. Eatough, L. Whiting, C. H. Bartholemew, C. L. Cluff, R. M. Izatt, J. J. Christensen, in *Conference on Trace Substances in Environmental Health, Proceedings*, D. D. Hemphill, Ed. (Univ. of Missouri Press, Columbia, 1974), vol. 8, p. 393.
21. F. Mukai, I. Hawryluk, R. Shapiro, *Biochem. Biophys. Res. Commun.* **39**, 983 (1970).
22. D. F. S. Natusch, *Environ. Health Perspect.*, in press.
23. R. C. Pierce and M. Katz, *Environ. Sci. Technol.* **10**, 45 (1976).
24. J. McCann and B. N. Ames, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 5153 (1975).
25. J. N. Pitts, Jr., D. Grosjean, T. M. Mischke, *Toxicol. Lett.*, in press; R. Talcott and E. Wei, *J. Natl. Cancer Inst.* **58**, 449 (1977).
26. Supported by the U.S. Energy Research and Development Administration. We thank Lloyd Davies and David Silberman for technical assistance, Dr. Marvin Goldman for advice and encouragement, Dr. Leon Rosenblatt for statistical support, and Drs. S. Book, O. Raabe, M. Shiffrine, and F. Wilson and Mr. J. Azevedo for critical reading of this manuscript.

17 June 1977; revised 15 August 1977

Pierce's Disease of Grapevines: Isolation of the Causal Bacterium

Abstract. *A Gram-negative, rod-shaped bacterium has been consistently isolated from grapevines with Pierce's disease. Grapevines inoculated with the bacterium developed Pierce's disease, and the bacterium was reisolated from the plants. The bacterium was serologically and ultrastructurally indistinguishable from the one in naturally infected plants, and also indistinguishable from a bacterium isolated from almonds with almond leaf scorch disease.*

The etiological agent of Pierce's disease (PD), an important and often devastating disease of grapevines (*Vitis vinifera* L.) (*1*), is also considered to cause alfalfa dwarf (*2*) and almond leaf scorch diseases (*3*). Prior to 1971, PD was considered to be a viral disease (*4*), but chemotherapy, thermotherapy, and electron microscopy subsequently implicated the "rickettsia-like" organism seen in the xylem vessels of diseased grapevines as the etiological agent (*2, 5, 6*). Many investigators have reported failure to isolate the PD pathogen from diseased plants using artificial media (*2, 6, 7*). Similar insect-vectored bacteria have recently been associated with a number of other plant diseases, and although determination of their pathogenicity and taxonomic position has been delayed by the inability of investigators to culture these bacteria, they apparently constitute a new group of plant pathogenic bacteria (*8*).

Recently, a Gram-positive, catalase-negative bacterium that could be isolated from infectious leafhopper vectors but not from diseased plants was reported to be the etiological agent of PD (*7*). However, this bacterium did not infect healthy plants following direct inoculation, and contradictory evidence as to its causal role has been reported (*9*).

We now report the consistent culture of a Gram-negative, catalase-positive bacterium from grapevines with PD, and evidence that this bacterium causes PD.

A rod-shaped, Gram-negative bacterium was first isolated on our JD-1 medium (Table 1) from grapevines experimentally inoculated by the leafhopper vector, *Hordnia circellata* (Baker). Initially, the inoculum was collected by centrifugation of sap from surface-sterilized petioles; in later isolations, inoculum was obtained by expressing sap from petioles with forceps. The inoculum was blotted directly from the petioles onto the media. Colonies on the JD-1 medium appeared after 2 to 3 weeks of aerobic incubation at 28°C. Progressive refinements in the culture medium shortened the necessary incubation period. On the JD-3 medium (Table 1), small but distinct colonies are visible without magnification within 6 days. Colonies are circular with entire margins, white, smooth, and convex, and reach a diameter up to 1.0 mm within 2 weeks.

We have consistently isolated the PD bacterium from diseased grapevines. In one isolation experiment, single petioles from 195 rooted cuttings of eight European grapevine varieties (Pinot Noir, Mission, Ruby Cabernet, Flora, Cabernet Sauvignon, White Riesling, Barbera, and Thompson Seedless) were used. Pierce's disease had been transmitted with leafhopper vectors to 116 plants. Of the 79 remaining plants categorized as healthy, 52 had been subjected to feeding by leafhoppers but did not develop PD, and 27 were noninoculated controls. Positive isolations on the JD-2 medium (Table 1) of the PD bacterium determined on the basis of colony characteristics were obtained from 97.4 percent (111/116) of the diseased plants. Only one plant in each group of healthy plants, or a total of 2.5 percent (2/79), yielded bacteria with colonies resembling those of the PD bacterium. Other bacteria were rarely isolated from diseased or healthy plants.

The pathogenicity of the PD bacterium was tested by inoculating green stem cuttings of the grapevine varieties Pinot Noir, Mission, and Ruby Cabernet. The upper end of each two- or three-node cutting with leaves intact was attached to a vacuum pump, and 0.1 to 0.2 ml of a turbid suspension of the PD bacterium (approximately 5×10^8 bacteria per milliliter) in sterile tap water was drawn into each cutting. Controls consisted of noninoculated cuttings and cuttings inoculated with sterile tap water alone, or with suspensions of *Erwinia amylovora* (8×10^8 bacteria per milliliter). After inoculation, the cuttings were rooted on a heated bench under intermittent mist for 14 days and transplanted. Typical PD symptoms (*1*) developed in 86 percent (43/50) of the cuttings inoculated with the PD bacterium within 2 to 4 months, and many of these plants died within 5 months. All 47 of the control plants remained healthy throughout the study. Colonies characteristic of the PD bacterium were reisolated from 35 of 36 in-