5000 and 4000 B.P., between 3740 and 500 B.P., and during the 19th century (*14*). The advance between 5000 and 4000 B.P. was the most extensive; the ice front reached some 10 km beyond its present position. These advances can be correlated with the three cool, moist intervals at Alerce subsequent to 4950 B.P.; the greatest advance corresponds with the heaviest precipitation. Elsewhere in the Southern Hemisphere all the observed Chilean neoglacial fluctuations appear parallel only with those in New Zealand (*5*); in Australia and New Guinea, neoglacial advances are thought to have occurred only during the past 4000 years (*4*). Data indicating glacial behavior during the cool and moist interval between 11,000 and 10,000 B.P. in southern Chile are scant (*15*), but relevant findings were reported for the Andes of Peru (*16*), the Carstensz Mountains of New Guinea (*4, 17*), the Arrowsmith Range of New Zealand (*18*), and South Georgia in the Subantarctic (*19*).

CALVIN J. HEUSSER
S. STEPHEN STREETER
Department of Biology, New York University, Tuxedo 10987

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12. The sites represent 109 surface samples; taxa are *Podocarpus nubigenus*, *Saxegothaea conspicua*, *Fitzroya cupressoides* (includes *Pilgerodendron uvifera*), *Drimys winteri*, *Weinmannia trichosperma*, *Pseudopanax laetevirens*, *Nothofagus dombeyi* type, *Eucryphia cordifolia*, *Tepualia stipularis*, Myrtaceae (other than *Tepualia*), Gramineae, Cyperaceae, Hydrangeaceae, Umbelliferae, Ericaceae-Empetraceae, Myzodendraceae, Gunneraceae, Compositae, Hymenophyllaceae, and Polypodiaceae [C. J. Heusser, *Pollen and Spores of Chile* (Univ. of Arizona Press, Tucson, 1971)].
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15. Mercer (*6*) found that glaciers had receded into the Chilean Andes before 12,500 B.P., were smaller at 11,000 B.P. than today, and did not advance again until the fifth millennium B.P. No conclusive evidence for an advance of Younger Dryas age (11,000 to 10,000 B.P.) was discovered. In contrast, climatic trends at Alerce in this time range, which emphasize lower temperature and increased precipitation, favored glacier alimentation and advance. Thus the data on glacier growth during Younger Dryas time, believed by Mercer to be equivocal, require further study.
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"Atmospheric" Epoxidation of Benzo[*a*]pyrene by Ozone: Formation of the Metabolite Benzo[*a*]pyrene-4,5-Oxide

Abstract. *Benzo*[a]*pyrene deposited on a glass fiber filter reacts rapidly in the dark or light with ambient levels of ozone to yield a mixture of products that display strong direct mutagenicity in the Ames assay. The major stable contributor to this activity has been identified as benzo*[a]*pyrene-4,5-oxide, a DNA-binding metabolite in biological systems, known to be a strong direct mutagen with* Salmonella typhimurium *strain TA98.*

Concern about the possible health hazard of air pollution by combustion-generated particulate organic matter (POM) historically has focused on its polycyclic aromatic hydrocarbon (PAH) content (*1*). Many members of this chemical class, such as benzo[*a*]pyrene (BP), are potent animal carcinogens (*2*) and are predominantly associated with small particles ($< 1\ \mu m$) that can be inhaled and deposited in lungs of humans (*3*).

Two lines of evidence suggest, however, that the possible health hazard presented by POM is not restricted to the PAH fraction. First, although organic extracts of POM from ambient air and the exhaust from spark ignition engines are known to be carcinogenic, their activity can be significantly greater than would be expected on the basis of their known PAH content (*1, 4*). Second, application of the Ames *Salmonella* mutagen assay to such extracts, as well as those from diesel particulates (*5*) and fly ash from coal-fired power plants (*6*), has demonstrated a significant level of direct mutagenicity (not requiring activation by mammalian metabolic enzymes), which is not ascribable to the promutagenic PAH.

Since the direct mutagenicity of ambient POM is of the frameshift type commonly associated with PAH metabolites (*7*), we suggested that it might be due in part to PAH derivatives formed during combustion, by reactions in the atmosphere subsequent to emission, or by processes that occur during collection of the POM on the commonly employed high-volume glass fiber filters. In experiments with simulated atmospheres, we demonstrated that directly mutagenic nitro derivatives of perylene and BP were produced when the parent PAH was deposited on such filters and exposed to airstreams containing 0.25 to 1.0 ppm NO_2 and traces of nitric acid (*8*).

In addition, production of a directly mutagenic product mixture from the reaction of BP exposed to levels of O_3 commonly encountered during air pollution episodes in many urban areas—for example, 0.1 to 0.2 ppm—was demonstrated (*9*). We now report that the major stable contributor to the direct activity of this product mixture is benzo[*a*]pyrene-4,5-oxide. This highly mutagenic (*10*) K-region epoxide has been identified as a DNA-binding metabolite of BP in several biological systems (*11*).

Chemical and microbiological procedures were carried out under darkroom illumination. Cleaned 8 by 10 inch Gelman AE borosilicate glass fiber filters (of the type commonly used for high-volume collection of ambient particulates) were coated by saturation with, and subsequent evaporation of, a dilute solution of BP (99+ percent) in toluene. Coated filters (~ 2 mg of BP per filter) were mounted in an apparatus that allowed exposure to a controlled atmosphere, either in the dark or under actinic ultraviolet illumination. Ozone was generated from pure, dry oxygen with a Welsbach model T-408 ozonizer and metered into a flow of filtered, purified air to provide low, stable O_3 concentrations at the filter surface. During a typical 4-hour exposure, the ozone concentration ranged from 190 to 210 ppb at a flow rate of 1.0 cubic foot per minute. Approximately 50 percent of the BP was decomposed in about 1 hour under these conditions in the dark, and approximately 80 percent over the entire 4-hour exposure.

Exposed filters were extracted by ultrasonic agitation in a mixture of methanol, toluene, and dichloromethane (1:1:1) and the extracts were filtered and

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Table 1. Comparison of spectral data for fraction 13-4 and benzo[*a*]pyrene-4,5-oxide (*14*). Values are mass-to-charge ratio (*m/e*), wavelength of maximum ultraviolet absorbance (λ_{max}), and fluorescence emission wavelength (λ_{em}).

Sample	Mass spectrum (*m/e*), 70 eV	Ultraviolet absorbance λ_{max} (nm)	Fluorescence λ_{em} (nm)
Fraction 13-4	268, 252, 250, 239, 237, 134	328, 315, 303, 275, 268 (ACN)	370, 390 (ACN)*
BP-4,5-oxide	268, 252, 250, 239, 237, 134, 119.5	328 (3.8), 315 (4.0), 303 (4.0), 275 (5.0), 268 (4.8)†	370, 390

*ACN, acetonitrile used as solvent to obtain spectra. †Numbers in parentheses are logarithms of extinction coefficients.

evaporated to dryness. Samples for microbiological testing were weighed, dissolved in dimethyl sulfoxide (DMSO), and quick-frozen in liquid N_2. Mutagenicity assays were conducted by the protocol of Ames *et al.* (*12*) with some recent refinements (*13*) that improve the reproducibility and sensitivity of the method for complex samples. The Ames *Salmonella typhimurium* strain TA98 was found to be the most suitable for this work because of its low spontaneous reversion frequency and high sensitivity to the BP-O_3 product mixture.

Our approach to isolation of the active components of the product mixture consisted of fractionation by reversed-phase chromatography followed by Ames testing of the resolved fractions. Initial separations were carried out with a methanol-water solvent system, which has been common practice in BP metabolism studies (*14, 15*). In this case, the highest levels of direct activity were exhibited by fractions containing the 1,6-, 3,6-, and 6,12-BP quinones. While these quinones are major products of BP ozonolysis (*16*), they are only slightly mutagenic (*17*). This suggested that a relatively small amount of a highly potent direct mutagen was present in the quinone fractions.

Fractionation of the product mixture with an acetonitrile-water solvent system, however, produced the chromatogram shown in Fig. 1a. Seventeen fractions were collected as indicated and assayed for direct mutagenicity to TA98 (Fig. 1b). Fraction 13, which had the highest specific activity at 85 revertants per microgram, was further resolved into several components (Fig. 1c); these were again tested microbiologically and examined by mass spectrometry. All but fraction 13-4 were inactive at the levels tested; 13-2 and 13-3 were identified as quinones, and 13-5 gave a mass spectrum corresponding to that of the major component of fraction 14 (molecular weight, 270).

The spectral characteristics of fraction 13-4 are presented in Table 1 together with those of authentic BP-4,5-oxide (*15, 18*). After brief irradiation (313 nm) in aqueous acetonitrile or treatment with acid, this sample also exhibited fluorescence at 425 and 445 nm, which was shifted to 540 and 570 nm on addition of KOH. These spectra are consistent with the presence of a mixture of 4- and 5-hydroxy-BP, known rearrangement products of the 4,5-epoxide (*19*). Photoinduced isomerization occurs in other arene oxides (*20*), and such photosensitivity is consistent with our observation that, although a slightly higher consumption of BP was obtained when ozone exposure was conducted under actinic illumination (94 percent versus 81 percent in the dark), the epoxide yield was reduced (0.07 versus 0.5 percent).

A solution of the epoxide produced in this reaction was quantitated by ultraviolet absorption (*15*) and plated with TA98 in the absence of S9 mix to produce the dose-response curve shown in Fig. 1d. The initial slope of 1600 revertants per microgram is in good agreement with the activity of the 4,5-oxide toward TA98 reported by other investigators (*10, 21*).

There are several alternative mechanisms for the production of the weakly carcinogenic (*22*) BP-4,5-oxide in this simulated atmospheric reaction. Direct epoxidation of BP by ozone, perhaps mediated by the glass surface, seems reasonable, since epoxidation is a common side reaction in the ozonolysis of sterically hindered *cis* olefins. Alterna-

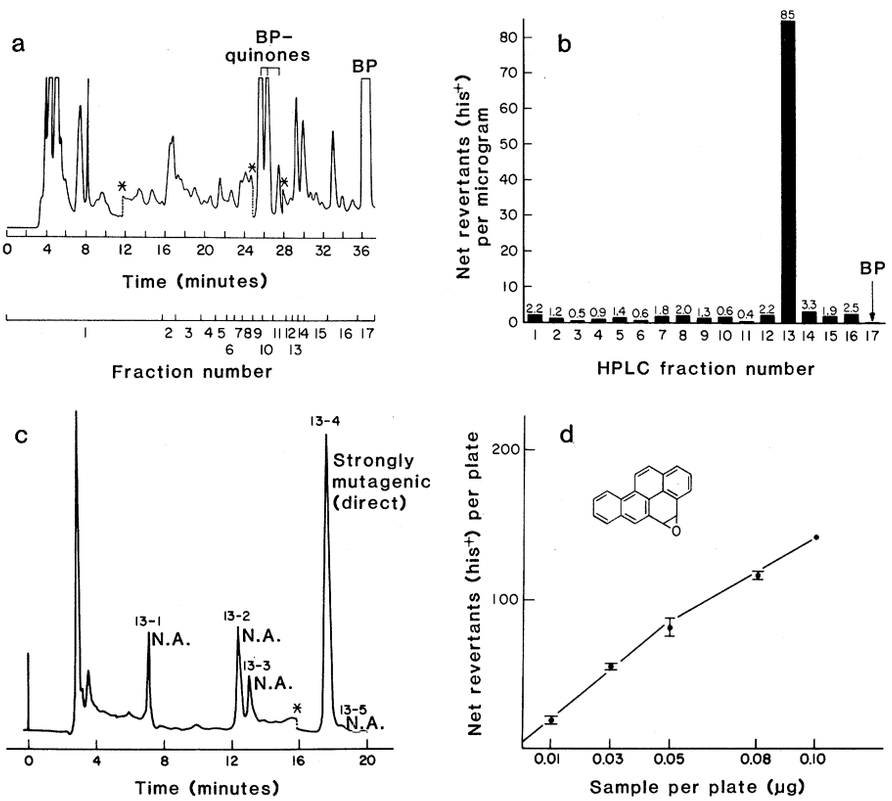

Fig. 1. (a) Preparative high-pressure liquid chromatographic (*HPLC*) separation of BP-O_3 products on two 7.8 by 300 mm μ-Bondapak C-18 columns at ambient temperature; 30-minute linear solvent program, 40 to 90 percent acetonitrile in water at 4.0 ml/min, ultraviolet absorbance detection (254 nm). Asterisk marks scale change. (b) Specific mutagenic activity of HPLC fractions of BP-O_3 products toward TA98, without activation. (c) Preparative HPLC separation of fraction 13; Altex Ultrasphere ODS 4.6 by 250 mm, 3:1 acetonitrile:water at 1.0 1.0 ml/min, ambient temperature, ultraviolet absorbance detection (254 mm); *N.A.*, no activity. (d) Dose-response curve for fraction 13-4 (TA98) without activation. Points represent the mean of revertant colony counts from duplicate plates (single plate at 0.1 μg). The spontaneous revertant background was 31 per plate.

tively, peroxidic intermediates produced in the BP-O_3 reaction might be involved in the epoxidation reaction, either on the filter during exposure or during work-up. To eliminate the last possibility, we included dimethyl sulfide in the extraction solvent; the epoxide yield was unaffected by the addition of this reducing agent (*23*).

From a chemical standpoint, the parallel between cellular metabolism of BP to produce the 4,5-oxide and formation of the 4,5-oxide in our simulated atmospheric system is intriguing in view of the proposed mechanism of PAH "activation" by the cytochrome P-450 mixed-function oxidases. Oxygen-atom transfer reactions are also known to be important features of the gas-phase chemistry of the polluted troposphere. Our results suggest that at least one such oxygen-atom transfer process may also occur in the troposphere through heterogeneous gas-particle "dark" reactions between ambient O_3 and BP. Thus such relatively stable arene oxides as BP-4,5-oxide may be capable of forming and surviving for significant time periods in the environmental cycle involving particulate emission, atmospheric transport and transformation, and ultimate deposition, whether on a filter or in the human lung. It is not known whether other direct mutagens, including the BP-7,8- and 9,10-oxides, are also formed in significant amounts in the BP-O_3 system, since these less stable isomers would probably not survive chromatography under the conditions we employed.

On the other hand, reactions of the type discussed may prove to be restricted to the surfaces of the collection device (such as a high-volume filter). In this case, analytical artifacts from these reactions may be expected to significantly affect chemical assays of the PAH content, as well as assays of the nature and degree of mutagenicity and carcinogenicity of the POM extracts. In the case of ambient POM collected on such glass fiber filters in regions that commonly experience even relatively light photochemical air pollution (for example, ~ 0.1 ppm O_3) the measured BP levels may be significantly low. Such errors will necessarily affect past and present epidemiology studies directed toward establishing correlations between ambient BP and, for example, the occurrence of lung cancer in the exposed human population (*1, 24*).

We believe heterogeneous ozonolysis and nitration processes may indeed occur both in the atmosphere and during the act of sampling, with relative efficiencies that depend on a number of factors including the specific PAH's involved, the physical and chemical nature of the particle surface, and whether photochemical processes are also involved. Indeed, it is interesting to speculate on whether ozonolysis can occur if inhaled photochemical smog interacts with BP adsorbed on particles already deposited in the lung.

With respect to possible health implications, at present we can only state that under our experimental conditions the BP-O_3 reaction produces a powerful, direct-acting mutagen that directly binds to DNA (*11*) and is a weak carcinogen on mouse skin (*22*). If the reaction also proves to occur in ambient POM, the possible health impact on the general population should be evaluated, since direct mutagens such as BP-4,5-oxide do not require metabolic activation to produce genetic damage.

JAMES N. PITTS, JR.
DAVID M. LOKENSGARD
PAUL S. RIPLEY
KAREL A. VAN CAUWENBERGHE*
LUK VAN VAECK*
Department of Chemistry and *Statewide Air Pollution Research Center, University of California, Riverside 92521*
STEVEN D. SHAFFER
ALAN J. THILL
WILLIAM L. BELSER, JR.
Department of Biology and *Statewide Air Pollution Research Center, University of California, Riverside*

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* Present address: University of Antwerp, Antwerp, Belgium.

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