Generation of Biologically Active Substances in a Natural Gas Flame

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Samples of gaseous and solid species taken from the central axis of a 1 megawatt heat-input natural gas flame were tested *in vitro* for mutagenic activity and teratogenic potential. Mutagenicity was determined by a *Salmonella typhimurium* forward mutation assay. Potential teratogenicity was indicated by the ability of samples to interfere with the attachment of mammalian cells to a lectin coated surface. Both the mutagenic and anti-attachment activities were found to peak in samples originating from the flame regions where the total polyaromatic compound (PAC) species concentration reached a maximum, indicating a strong correlation between PAC presence in the samples and biological activity. Additional anti-attachment activity was found close to the injection nozzle. No biologically active material was detected beyond the luminous portion of the flame.

Introduction

A multidisciplinary program with participation from the Departments of Chemistry, Chemical Engineering, and Applied Biological Sciences on the health effects of fossil fuel combustion products is in progress at MIT. In the 1.2 × 1.2 × 4.5 m combustion tunnel of the MIT Combustion Research Facility (CRF), the fundamentals of industrial type flames can be studied by detailed exploration of the various flame regions. The thermal and chemical environment and the residence times of industrial flames are closely simulated in this facility. By determining the spatial distributions of gas temperature, velocity, and chemical species in the flame, the reactions of individual chemical compounds can be followed.

In this paper we report on bioactive chemical species formation and destruction within a 1 megawatt (MW) thermal input methane-air turbulent flame. Gaseous and solid (soot) samples from within the flame were examined for mutagenic activity and for potential teratogenic activity using two simple *in vitro* assay systems. Samples taken from narrow regions of the flame were found to contain mutagens; other nearby regions contained potential teratogens. No toxic activity was detected in samples collected beyond the luminous portion of the flame.

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Methods

Combustion

The 1.2 × 1.2 × 4.5 combustion tunnel at the MIT CRF was operated at 1 megawatt heat input. The fuel used in the studies reported here was commercial natural gas (97% CH_4, 2% CO_2, with the remainder as light hydrocarbons). Experimental input variables are listed in Table 1. Figure 1 is a schematic diagram of the luminous portion of the flame. Conditions were chosen to correspond to those of "staged combustion," a method used to minimize NO_X emission (1). In staged combustion, fuel was first mixed with a fraction of the combustion air (here 2/3) required for complete combustion. Thus, the initial flame region was fuel rich. The remainder of the air was added 452 cm from the injection nozzle, well beyond the luminous portion of the flame. While staged combustion suppressed nitrogen oxide formation and emission, it favored high molecular weight hydrocarbon and soot formation in the fuel-rich first stage of the flame. NO_X concentrations did not exceed 23 ppm at any point in the flame (2).

Sampling

Samples of gaseous and solid (soot) species were taken from 60 points in the flame. We report here on eight samples collected at several points along the axis of the flame. The flame axis is the horizontal line originating at the center of the injection nozzle. Gas temperature and velocities were also measured at these points. The

sampling system used is shown schematically in Figure 2. A water-cooled probe, described elsewhere (*3*), was inserted into the flame through sampling ports in the wall of the combustion tunnel. Gas and solid (soot) samples entering the probe were mixed with a stream of cold water at the tip of the probe to prevent further reactions. The waterborne sampling stream was then passed through a glass fiber filter to retain soot particulates and then through a 50 hydrocarbon resin (XAD-2) absorber to retain condensable hydrocarbons. Carrier water was collected in a cooled trap and accumulated to about 300 mL in a typical run. Gas temperature, flow, and pressure were monitored to permit calculation of in-flame combustion product concentrations.

Sample Extraction

The filter and XAD-2 resin were placed in the same Soxhlet apparatus and extracted with methylene chlo-

Table 1. Furnace operating parameters.

Flame tunnel: 1.2 m × 1.2 m × 4.5 m
Natural gas composition:

Compound	Mole fraction, %
H_2	0.0
N_2	0.0
CH_4	97.0
CO_2	1.8
C_2H_2	0.0
C_2H_4	0.1
C_2H_6	1.1
C_3H_6	0.1
C_3H_8	0.2

Steady state achieved in 4–5 hr
Injection parameters:
    Gas: 1.25 kg/min (2.76 lb/min)
    Air: 15 kg/min (33 lb/min)
    Initial fuel equivalence ratio: 1.3 (fuel rich)
Flame temperature: 1300°K–1630°K

FIGURE 2. Schematic diagram of sampling train used in this study. Combustion products collected with a water-cooled probe and quenched with a stream of water were drawn through a 183 mm glass fiber filter and 50 g XAD-2 resin. The water was collected in a cooled trap.
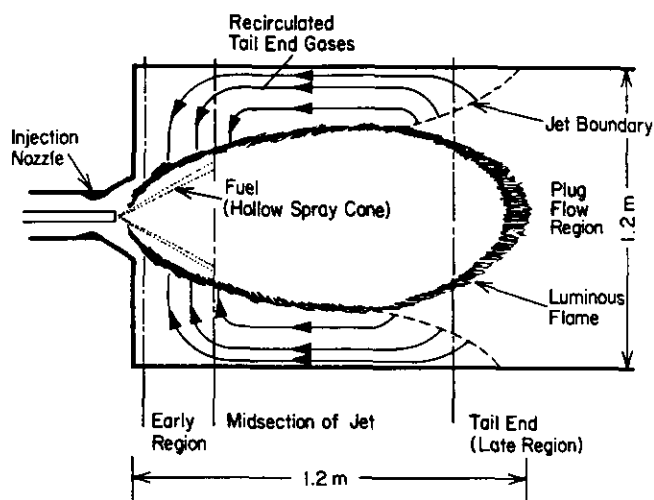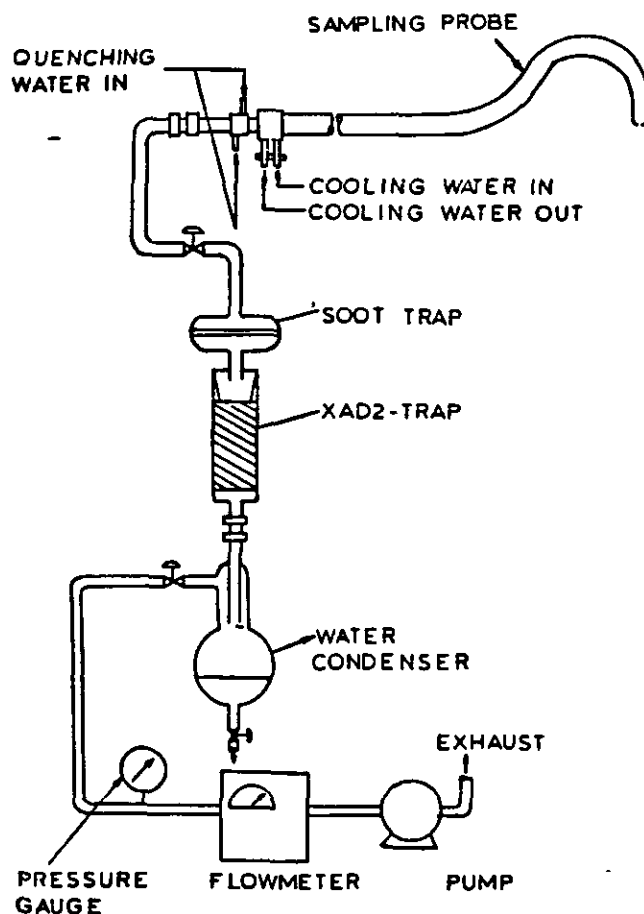
FIGURE 1. Schematic diagram of the luminous portion of the natural gas flame studied here. Samples were taken at several points along the flame axis; a horizontal line extending from the center of the injection nozzle to the right.

ride for 20 hr. Carrier water was extracted separately with this solvent. Both methylene chloride extracts were combined and concentrated from about 400 mL to roughly 7 mL in a specially designed vacuum condenser apparatus (*4*). Low molecular weight aromatics were efficiently retained in this concentrator. For example, there was 80% recovery of naphthalene following concentration. Polyaromatic compound (PAC) concentration was estimated from gas chromatographic analysis. Since methylene chloride was very toxic to bacterial and mammalian cells, the concentrated samples were removed from the methylene chloride and placed in dimethyl sulfoxide, a less toxic solvent. Aliquots of the methylene chloride solution were mixed with appropriate volumes of DMSO and placed in a stream of nitrogen for roughly 20 min to vaporize the more volatile methylene chloride. In several samples, material exchanged from methylene chloride was only slightly soluble in DMSO. These samples were tested to their solubility limit. It should be emphasized that only methylene chloride-soluble materials were examined. Soot particles constituted the principal material collected beyond 100

cm from the injection nozzle. Little methylene chloride-soluble material was present on these particles.

Bacterial Mutagenesis

Mutagenic activity was tested using the *Salmonella typhimurium* forward mutation assay of Skopek et al. (*5,6*) with and without Aroclor 1254-induced postmitochondrial supernatant (PMS). This assay measured the induced 8-azaguanine resistant mutant fraction following a 2-hr, 37°C treatment with test agent. Survival following treatment was measured so a true mutation fraction could be calculated. Significant mutagenic activity was judged to be present in a sample if treatment increased the mutation fraction above the historical 99% upper confidence limit for untreated cultures. There is no single parameter that can describe a complex dose-response relationship between test agent concentration and induced mutant fraction; however, for convenience we have chosen to use the maximum slope from dose-response curves as a measure of mutagenic activity in a sample. All samples were tested to a concentration of 300 μg/mL or their maximum solubility, which was judged visually.

Teratogen Assay

Potential teratogenicity was measured using the attachment inhibition assay system of Braun et al. (*7–9*). Briefly, this system measures the ability of murine ascites cells treated with test agent for 2 hr at 37°C in physiological saline to attach to concanavalin A coated surfaces. Samples inhibiting attachment without cytotoxicity, as determined by trypan blue exclusion, were deemed to be potentially teratogenic. In the assay, ascites tumor cells, labeled *in vitro* with tritiated thymidine, washed, and resuspended in phosphate-buffered saline (PBS: 0.137 M NaCl, 2.7 mM KCl, 2.5 mM sodium phosphate buffer, pH 7.4), were incubated for 2 hr with aliquots of flame extract in PBS at 37°C, and poured over three or four 1.25-cm diameter polyethylene disks coated with concanavalin A. The cells were permitted to sediment onto the disks for 20 min at room temperature, the disks were removed and washed in PBS, and the attached tritium counts were measured in a liquid scintillation counter. Attachment is thought to model morphogenic cell-cell or cell-extracellular matrix interactions. Agents interfering with attachment may also interfere with these morphogenic interactions in the developing embryo and hence lead to congenital malformations.

Although there is evidence that some teratogens require metabolic modification for their activity (*10,11*), most appear to act directly on the cell surface (*8*). Preliminary studies with combustion-derived PAC indicate that metabolic activation does not increase their activity (Braun and Harding, unpublished data).

It should be noted that many mutagenic agents are also animal teratogens (*12*). Thus, both the mutagenic

activity and the activity inhibiting attachment represent potential teratogenic activity in a sample.

Results

Figure 3 shows the relation between the amount of methylene chloride-extractable material collected by the sampling train and the sampling position. Extract weight is expressed in terms of gas volume corrected to normal temperature and pressure in units of normal cubic meters. Distance was measured from the fuel injection nozzle. The chemical composition of each sample was determined by gas chromatographic analysis and has been reported elsewhere (*13,14*). Samples of adequate size for biological testing were obtained between 44 cm and 255 cm. A broad peak of extractable material was found from 99 cm to 136 cm. Beyond 136 cm, nearly all the material collected in the sampling train was solid (soot). We were unable to detect any PAC in the methylene chloride extract of these soot particles.

Figure 4 shows that the specific mutagenic activity varied widely with sampling position. A peak of PMS-independent mutagenic activity at 105 cm was narrower than the peak of extractable material at this position (Fig. 3). Material collected at 117 cm had considerably less specific mutagenic activity than that collected at 105 cm. A much smaller but broader peak of mutagenic activity detected in the presence of PMS was centered at 136 cm.

When samples were tested for their ability to inhibit tumor cell attachment to concanavalin A-coated plastic, a different pattern of specific activity was found (Fig. 5). Inhibitory activity peaked at 117 cm, somewhat beyond the peak of mutagenic activity at 105 cm. Both the mutagenic and the anti-attachment activity peaks lay within the broad peak of extractable material at 99 cm to 136 cm.

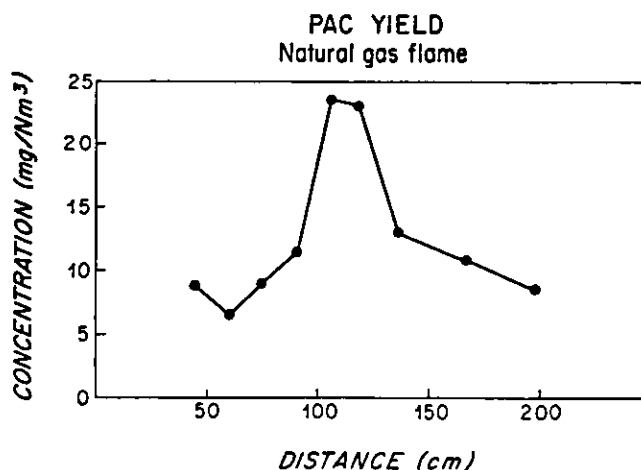In addition to the peak of specific anti-attachment

FIGURE 3. Amount of methylene chloride-extractable material collected vs. sampling position. Extract concentration is expressed in terms of gas volume collected, adjusted to standard temperature and pressure.

MUTAGENIC ACTIVITY
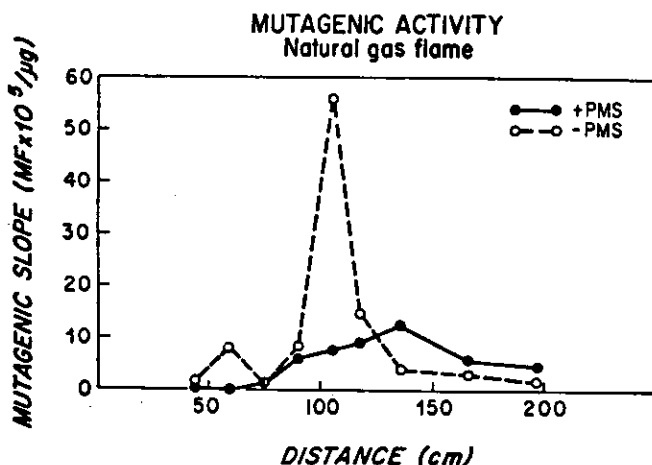### Natural gas flame

FIGURE 4. Specific mutagenic activity of methylene chloride extracts vs. sampling position. As discussed under "Methods," the maximum slope of mutagenic activity vs. extract concentration has been used as a measure of mutagenic activity in each sample. Samples were tested in the *Salmonella typhimurium* forward mutation assay with (+PMS, ●) and without (-PMS, ○) metabolic activation.
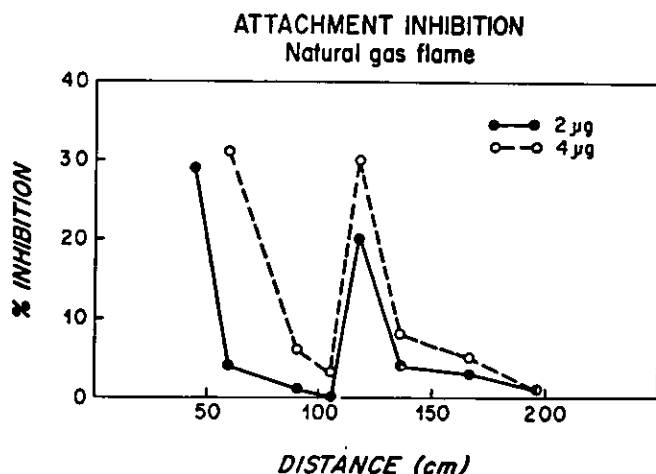
ATTACHMENT INHIBITION
Natural gas flame

FIGURE 5. Ability of flame extracts to inhibit the attachment of tumor cells to concanavalin A-coated surfaces. Two (●) and 4 (○) μg of each extract were incubated with ascites tumor cells (DNA labeled with tritium) for 2 hr (37°C) and poured over disks coated with concanavalin A. After 20 min, the disks were removed, washed, and counted to determine the number of cells attached. The inhibitory effect of treatment is plotted vs. sampling position.

activity at 117 cm, there was increased activity near the fuel injection nozzle. There was little mutagenic activity in these early samples. As no samples were taken at less than 44 cm, it was not possible to document the development of anti-attachment activity from methane. Gas chromatographic analysis indicated almost no PAC species in the 44 cm sample. However, it is likely that low molecular weight hydrocarbons were present but not detected with conventional gas chromatographic techniques.

The total yield of mutagenic activity as a function of axial position is more relevant to the issue of health

impact than are the specific activities described thus far. An estimate of the total yield can be derived by multiplying the specific activity shown in Figure 4 by the yield of extractable material per standard cubic meter depicted in Figure 3. This total activity is plotted in Figure 6. Because the greatest yields of PAC and specific activity coincide, the total activity profile is similar to the specific activity pattern (Fig. 4). A similar calculation using the specific anti-attachment activity data of Figure 5 leads to the total activity pattern shown in Figure 7. Because little material was collected near the injection nozzle, the relative yield of inhibitory ac-

MUTAGENICITY x YIELD
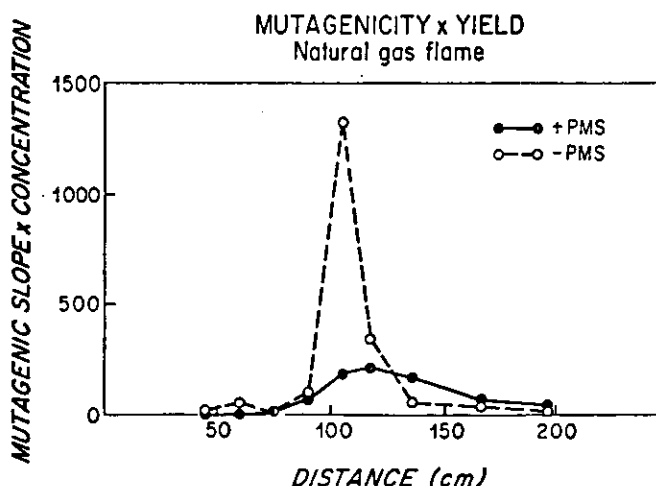### Natural gas flame

FIGURE 6. Total mutagenic activity vs. sampling position. The specific mutagenic activity shown in Fig. 4 has been multiplied by the total extractable material in Fig. 3.

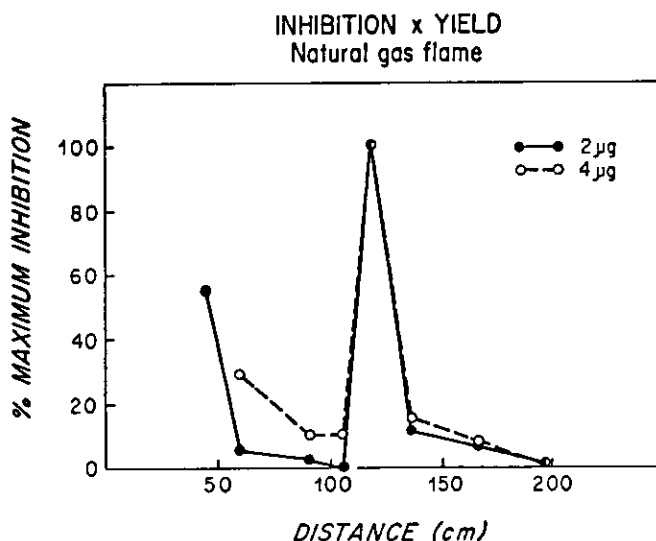## INHIBITION x YIELD
Natural gas flame

FIGURE 7. Total anti-attachment activity vs. sampling position. The anti-attachment activity in Fig. 5 has been multiplied by the amount of material extracted in Fig. 3 to give the total anti-attachment yield. To facilitate comparison of the 2 (●) and 4 (○) μg results, the total yields have been plotted as a percentage of the maximum anti-attachment activity.

tivity at 44 cm was less than would be expected from its specific activity.

Discussion

During combustion, a complex sequence of chemical reactions takes place. Models of detailed chemical kinetic schemes for the transformation of hydrocarbons under pyrolytic and oxidative flame conditions have been developed for simple hydrocarbons such as methane (15) and benzene (16). These models arise from experimental studies with laboratory systems using premixed reactants and laminar flow flames. Despite important differences between laminar premixed and turbulent postmixed flames, the reaction pathways, for simple fuels, have been found to be similar (3,13,14).

From studies of premixed laminar flow flames, a scheme for the methane-oxygen reaction has been reported by Warnatz (15) and is shown in Figure 8. According to this scheme, oxidative thermal methane dehydrogenation is followed by a series of radical reactions whose most important products are acetylene, polyacetylenes, and the 1,3-butadienyl radical. The importance of the 1,3-butadienyl radical in the present context is its ability to form aromatic species by reacting with
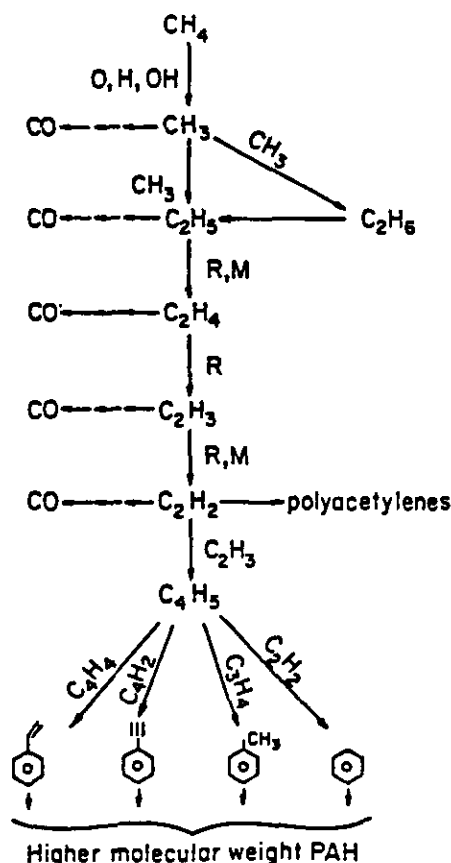

FIGURE 8. Initial reactions thought to lead to the formation of PAC from methane during combustion. This scheme is based on a proposal by Warnatz (15). R: Radical hydrogen acceptor, M: energy absorbing molecule.

acetylenic compounds. Cole (17) has found good agreement between measured rates of benzene, toluene, phenylacetylene, and styrene formation and rates predicted by addition of C_2H_2, C_3H_4, C_4H_2, and C_4H_4, respectively, to the 1,3-butadienyl radical.

As increasingly higher molecular weight PAC compounds are formed by addition, soot particles begin to nucleate. Once nucleation is initiated, soot growth is rapid, with about every tenth collision between particle and hydrocarbon depositing a carbon atom (18). In theory, soot can act as a carrier of residual PAC if vapor phase PAC are absorbed on the large surface area of soot particles as combustion products are cooled by contact with cool surfaces and gases. However, soot collected at temperatures higher than 300°C was found to contain no methylene chloride-extractable compounds.

This theoretical sequence of chemical events was reflected in the spatial distribution of specific compound flux along the flame axis. Early synthetic events occurred near the injection nozzle, while sooting and late degradative reactions took place at the flame tail.

The dominant mutagenic activity detected in the flame did not require metabolic activation (Fig. 4). Pure polyaromatic hydrocarbons (PAH) require activation for mutagenicity. It is therefore likely that the direct acting mutagenic activity is due to polycyclic aromatics containing a heteroatom. Under the fuel-rich condition of this flame, a strong candidate for the heteroatom in these mutagens is nitrogen. A wide variety of nitroaromatic compounds are stable, direct-acting bacterial mutagens. For example, polynitroaromatics, such as 1,8 dinitropyrene, are highly mutagenic in the absence of metabolic activation. Indeed, the addition of post-mitochondrial supernatant and cofactors inactivates many of these agents. How can nitrogen containing PAC arise? As the natural gas fuel contains only hydrocarbons (Table 1), the heteroatom must arise from reactions involving species such as NH_3, NH_2, HCN and NO_x, derived from atmospheric N_2. Whether these reactions took place in the gas phase of the flame, or soot particles suspended in the flame, or on the sample collection apparatus is unknown.

We favor the hypothesis that the direct acting mutagens were formed in the flame. Although the overall fuel equivalence ratio was 1.3 (slightly fuel rich), rapid oxygen consumption in the central region of the flame, where samples were taken, resulted in a far higher equivalence ratio (> 3). Under these extremely oxygen poor conditions, nitric oxide, ammonia, HCN, and hydrocarbons, including PAC radicals, exist in abundance (19). Reactions between the PAC radicals and ammonia or HCN species might result in nitrogenated PAC, compounds which in turn might cause the PMS-independent mutagenic activity. Since neither the mutagenic species nor the reaction kinetics involved are known, an in-flame mechanism remains purely speculative.

A plausible alternative origin for the PMS-independent mutagens involves reactions outside the flame, perhaps in the sampling train. At NO_x concentrations greater than 1 ppm, Pitts et al. (20) have shown that

reactions between immobilized PAH and NO_2 occur at room temperature. Although the bulk of the mutagenic species thus generated required exogenous metabolic activation, Pitts found that some reaction products were PMS-independent mutagens. Solid phase reactions between PAH and NO_X could occur either in flame on soot particles or on the sampling train filter or XAD-2 resin. Reactions on soot particles seem unlikely since soot was not detected at less than 136 cm into the flame, and PMS-independent mutagenic activity peaked at 105 cm.

A recent report by Brorstrom-Lunden and Lindskog (21) indicates that rapid chemical changes in PAH bound to XAD-2 resin take place when they are subjected to gaseous NO_X at 150°C. While conditions in the current study (low temperature and water-gas born NO_X) were quite different from those of the Brorstrom-Lunden and Linskog study, the possibility of mutagenic PAC formation in the sampling train cannot be excluded.

No matter where the PMS-independent mutagens arise, they constitute a potential environmental hazard. If mutagens originate from vapor phase reactions within the flame, they may be emitted intact. If solid phase reactions between PAH and NO_X are responsible for the production of mutagens, these reactions may occur at any surface beyond the flame having sufficiently low temperatures to allow PAH condensation. Possible sites of mutagen formulation are suspended soot particles, heat exchangers, internal surfaces of the exhaust system, or particulate collectors.

The analysis of PMS-independent mutagen formation is impossible without first directly identifying the chemical species responsible. It is clear that these mutagens are a minor component of PAC collected. Gas chromatographic/mass spectroscopic analyses do not disclose any compounds known to be direct acting mutagens. It appears that the bacterial mutation assay is sensitive to small quantities of highly mutagenic chemicals obscured by large amounts of relatively inert compounds. The eventual identification of the mutagens in these samples will require multiple fractionation steps monitored by bacterial mutagen assays.

Potentially teratogenic compounds that inhibited the attachment of mammalian cells to concanavalin A-coated plastic were found at 136 cm, coincident with the peak of PMS-dependent mutagens. Presumably, this activity was due to the PAC found in the region. The nature of inhibitory compounds in a second region of activity near the nozzle is unknown. It is possible this early inhibitory activity was due to linear hydrocarbons such as polyacetylenes rather than to aromatic species. Linear hydrocarbons have been found to inhibit attachment (22).

In an ideal flame, complete fuel combustion yields only CO_2 and H_2O. However, in reality, design constraints, flame instability, and uneven fuel-oxygen mixing result in the emission of small quantities of incomplete combustion products. Cooling (or "quenching") the flame in regions of PAC synthesis inhibits destruction of PAC and leads to their emission. As many PACs are toxic, combustion-mediated synthesis of PAC becomes of pub-

lic health interest. Even fractionally small amounts of PAC emissions from large furnaces may become substantial health hazards.

The samples tested here were obtained on the axis of the flame. Because turbulent jet diffusion flames are not two dimensional, it will be important to examine the radial distribution of mutagenic and anti-attachment activity. As a three-dimensional picture of toxic product synthesis and destruction becomes available, it will become possible to understand how PACs escape from industrial flames and to suggest novel combustor designs which reduce toxic compound emission.

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