

GEOGRAPHICAL AND TEMPORAL DISTRIBUTION  
OF ATMOSPHERIC MUTAGENS IN CALIFORNIA

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## Abstract

The Statewide Air Pollution Research Center has undertaken an assessment of the mutagenic potency, as determined by the Ames Salmonella/mammalian-microsome mutagenicity assay, of suspended particulate matter in the South Coast Air Basin (SCAB) of Southern California with support from the California Air Resources Board. During the period covered by this report, we have:

- Developed, and incorporated into our Ames test protocol, refinements which have resulted in a reduction of variability in the results sufficient to allow meaningful quantitative comparisons to be made among ambient particulate sample extracts.

- Examined the influence of filter material upon the chemical composition and mutagenic activity of particulate samples collected by high-volume filtration. For collection periods of greater than 40 hours, samples obtained using glass, quartz, or Teflon-impregnated glass filters show no consistent or significant differences in measured mutagenic activity, polynuclear aromatic hydrocarbon content, or HPLC profile. However, Teflon-impregnated glass fiber filters did show marginally improved extraction efficiency of organics relative to glass or quartz filters.

- Evaluated the methods used for the extraction of organic mutagens from ambient particulate, extract sample handling, and Ames testing of the extract, in terms of precision and accuracy. We have determined that, in parallel processing of equivalent samples, over 90% of the extractable mutagenic material is included in our "total extract" and that a precision of  $\pm 15\%$  in terms of measured activity is attainable.

- Carried out an assay of the average mutagen content, as determined by the response of strain TA98, of ambient particulate at eight locations during three consecutive day and night periods (7/11/79 to 7/14/79) in the South Coast Air Basin. Values of airborne mutagen density ranged from 8 to 45 revertants/m<sup>3</sup> in the absence of metabolic activation, and from 10 to 62 revertants/m<sup>3</sup> with metabolic activation. No correlation was apparent between the air quality and meteorological data obtained at each sampling site and the measured mutagenicities of the samples although the impacts of traffic density, industrial emissions and transport phenomena appeared to be clearly reflected in the data.



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The statements and conclusions in this report are those of the contractor and not necessarily those of the California Air Resources Board. The mention of commercial products, their source or their use in connection with material reported herein is not to be construed as either an actual or implied endorsement of such products.



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## I. EXECUTIVE SUMMARY

The Statewide Air Pollution Research Center has undertaken an assessment of the mutagenic potency, as determined by the Ames Salmonella/mammalian-microsome mutagenicity assay, of suspended particulate matter in the South Coast Air Basin (SCAB) of Southern California with support from the California Air Resources Board. The primary goals of this program are:

- To develop and employ accurate and reproducible methods for quantitation of the mutagenicity associated with ambient particulate as a function of time of day, season, and location within the SCAB.
- To gather, concurrent with particulate collections, air quality and meteorological data at each collection site.
- To identify those chemical species responsible for the observed mutagenic activity of urban particulate extracts with emphasis on identifying direct mutagens (which do not require activation by mammalian metabolic enzyme systems in the Salmonella mutagenicity assay).

Significant progress has been made in the development and validation of the methods which have been adopted for the performance of these tasks, and a preliminary determination of the geographical distribution and diurnal variation of particulate-associated mutagenicity has been carried out. The following pages are an Executive Summary of the results for year one; Sections II through VIII provide details of the work carried out.

### A. 1978 Filter Media Comparison Study (Section II)

Preliminary validations of the methods chosen for use in this study were necessary prior to initiation of collections for quantitative measurements in the field. Several variables may be capable of affecting the results of the mutagenicity testing in an artifactual way, leading either to positive or negative errors. One of these variables is the composition of the filtering medium used in high volume sampling. Interaction of the filter surface with the collected particulate in the presence of reactive pollutant gases and vapor-phase organics could conceivably result in surface-catalyzed chemistry of adsorption/desorption phenomena which would result in alteration of the collected material during the sampling period. In order to assess the magnitude of such effects on mutagenicity measurements we initiated, in 1978, a parallel collection of ambient

particulate on several different filter types (quartz, glass, Teflon-coated glass, and pure Teflon) and performed subsequent mutagenic assays of the extract, and of the acidic, basic, and neutral ether-soluble fractions derived from them. Interpretation of the results of this study was rendered impossible by non-linearity and irreproducibility in the dose-response curves obtained using the standard protocol for the Ames Salmonella/mammalian-microsome mutagenicity test (Ames et al., 1973; Ames and McCann, 1975; Ames, 1979). Refinement of the mutagen testing procedures was undertaken as a necessary preliminary step to further work in this area.

B. Quantification of the Ames Salmonella/Mammalian-Microsome Assay  
(Section III)

Our early experiments using this assay showed a high level of variability in the results. This inconsistency was attributed to variability in the test itself, and seriously limited our ability to quantitatively determine the mutagenic activity of environmental samples. We have made an extensive investigation of the factors responsible for the test's poor precision and have developed an improved methodology.

Using the conventional protocol (Ames et al., 1975), we obtained results in which the maximum plate counts were as much as 300% of the minimum plate counts at a constant mutagen dose level. Our new protocol has resulted in about a one order of magnitude reduction in the plate-to-plate variability (normally, replicate plate counts are within  $\pm 10\%$ ), as well as in greater consistency from experiment to experiment. In seeking improvements, we have attempted to avoid changes in the basic procedure which would alter its spectrum of sensitivity or otherwise render it incompatible with previous tests.

The two most important innovations arising from our detailed study are (a) the introduction of precise temperature control during incubation and (b) the development of a method of spreading the top agar containing the test sample and bacteria in a uniformly distributed layer over the base agar surface.

Other investigated sources of variability and procedure included the following: (a) effect of varying soft-agar temperatures on survival of cells; (b) the effect of the number of cells plated on the number of revertants per plate for standard mutagens; (c) the effect of base agar

volume on the mutagenic activity of diffusible and relatively nondiffusible mutagens; (d) automated colony counter calibration effects; (e) S9 suppression and optimal concentrations.

From these investigations we have arrived at the following modifications in the Ames assay for use in our protocol:

(1) The top agar should be leveled on the test plate by mechanical vibration.

(2) Test plates should be incubated in an incubator having a small ( $< \pm 0.25^{\circ}\text{C}$ ) temperature gradient for at least 63 hours.

(3) Soft agar ingredients should be mixed for at least three seconds with a mechanical vortex mixer.

(4) Cells should not be held in molten top agar any longer than necessary.

(5) An overnight culture should be adjusted to a cell density of  $\sim 1 \times 10^8$  cells/plate.

(6) Test-plate base agar volume should be consistent. We have found 20 ml machine-poured plates to be optimum.

(7) Automatic colony counters should be focused, adjusted for maximum sensitivity and calibrated regularly, with calibration verified with each experiment.

(8) For screening samples of unknown mutagenicity, we recommend the use of a low (2% v/v), a medium (10% v/v) and a high (up to 40% v/v) concentration of S9, if sample size permits.

(9) Preliminary testing should be carried out on duplicate plates with the five major tester strains. Each sample is screened at four concentrations over a 1000-fold range. A minimum of three S9 concentrations are used if sample size permits.

(10) Quantitative testing is performed on triplicate plates with the most responsive strain(s) and optimum S9 concentration. Sample concentrations are chosen to best characterize the linear portion of the dose-response curve as the sample size permits. These concentrations are usually within one order of magnitude of those which exhibit the highest activity as determined by the preliminary test.

(11) It is essential to run a full screen of controls every time samples are tested and to characterize the activity of ambient particulate extracts relative to known mutagenic standards.

We believe the modifications we have made render the Ames method adequately quantitative to accurately characterize the relative mutagenic potency of ambient particulate extracts.

C. 1979 Filter Comparison Study (Section IV)

The filter comparison study was resumed using an improved Ames test protocol. Three independent experiments were performed with three sample sets obtained by relatively long term (40-70 hours) simultaneous collections using sixteen high-volume samplers located at Harvey Mudd College in Claremont. Filter types were compared in sets of four. The filters examined included the glass fiber filters recommended by the EPA in 1978 and 1979, used as received; Gelman A-E glass fiber filters, which had been precleaned by extraction with organic solvents followed by oven firing at 450°C; quartz fiber filters, heated to 450°C for five hours; and Teflon-impregnated glass fiber filters, washed with organic solvents and vacuum dried. Samples collected by electrostatic precipitation were also included in two of the comparisons. The experimental approach involved extraction of the samples collected on each filter type during each collection period and separation of the extracts into ether-soluble acidic, basic, and neutral fractions by a liquid-liquid extractive separation. The ether-soluble fractions derived from these samples were weighed and their relative mutagenic activities were determined by the Ames test, both in the presence and absence of mammalian metabolic enzymes. In addition, chromatographic profiles (as determined by HPLC with UV detection in a reversed phase system) and levels of some selected polynuclear aromatics (as determined by GC-MS using selective ion detection) were obtained from the neutral fractions.

The three independent data sets which were generated contained the total particulate weight collected on each filter type, the weights of each fraction and of the total ether-soluble extract, the mutagenic activity of each fraction, a chromatogram of each neutral fraction, and the concentrations of the more abundant polynuclear aromatic hydrocarbons in the neutral fractions. No significant or reproducible difference among the samples collected on these filter types was observed by any of these techniques. However, the Teflon-impregnated glass fiber filters did show a

consistently higher yield of organic material from the extraction and fractionation process, indicating that the expected decrease in adsorptive properties of this material relative to glass or quartz may result in a higher extraction efficiency in the recovery of particulate organic matter (POM) from this filter type.

D. Examinations of the Reproducibility of the SAPRC Extraction and Fractionation Procedure (Section V)

The improvements in the Ames assay described in Section III of this report have enabled an assessment of the reproducibility of the methods used in this geographical distribution study, with the recognition that a part of the value of this work will be determined by the repeatability of the ambient mutagen assay by other workers.

The extraction method used in this study (ultrasonic agitation of the loaded filter with a solvent composed of equal parts of methanol, dichloromethane and benzene) has previously been demonstrated to remove more than 95% of the organic carbon (as determined by combustion analysis) from typical 24-hour hi-volume particulate samples taken in polluted air (Grosjean, 1975). We have recently evaluated the extraction efficiency of this technique with respect to mutagenic activity as determined by the Ames test, after substitution of toluene for benzene in the solvent mixture. The mutagen extraction efficiency of our method is comparable to that determined earlier for organic carbon; more than 90% of the extractable mutagenic activity, both direct and activatable, is included in the sample obtained from a single extraction.

Experiments designed to evaluate the extraction, sample handling, and Ames assay procedures used for quantitating "total" extract mutagenicity have demonstrated a high degree of reproducibility. Standard deviations for the entire process of better than 10% from four identical replicate samples have been obtained using these methods.

The reproducibility of acid/base/neutral fractionation procedures as applied to the extracts used in the filter comparison studies, while acceptable on a fractional mass basis, has been inferior in terms of the mutagenicity assay to those results obtained through testing the total extracts. We have not yet been able to determine the effect of such sample fractionation treatments upon the mutagen content of the ambient particulate

extracts, i.e., whether mutagens may be formed, deactivated, or lost in aqueous phases during the process. In addition, the inclusion of a fractionation step in a mutagen assay of this type necessarily increases the time needed for processing and thus leads to an undesirable variation in the extract histories within the large experimental data sets. On the basis of these considerations, we have chosen to perform the geographical distribution mutagen assay on the total extract of the sample filters rather than on those derived from a chemical fractionation procedure.

E. High Performance Liquid Chromatography Separation and Chemical Analysis of Ambient Particulate Extracts (Section VI)

A Waters high performance liquid chromatograph (HPLC) equipped with solvent composition gradient programming capability has been used in the reversed-phase mode (C-18  $\mu$ -bondapack columns) for the separation of neutral components of ambient particulate extracts collected in the SCAB. The resolved fractions exiting the HPLC have been tested for mutagenicity, and the most active fractions have been further purified and characterized by spectroscopic methods. While the active fractions obtained by this technique consist of complex mixtures, some conclusions can be drawn from their chromatographic and spectroscopic properties. The bulk of the direct activity displayed by the neutral fraction of the extracts is attributable to compounds of low to moderate polarity, while most activatable mutagenicity is localized in HPLC fractions containing polycyclic aromatic hydrocarbons. The spectral data obtained from fractions exhibiting high direct activity indicate that this activity may be due to the presence of PAH oxidation products such as epoxides, phenols, dialdehydes, lactones, and quinones.

F. Geographical Distribution of Mutagenic Activity Associated with Airborne Particulate Matter (Section VII)

Most phases of the first ambient mutagen analysis for this program have been completed, including collection, extraction and Ames assay of the samples, and compilation of air quality parameters and meteorologic data. Any search for statistically reliable correlations of the measured mutagenicities with the other variables in the air pollution system will require additional studies during the remainder of this program. However, some preliminary conclusions regarding trends in the relative levels of

mutagenicity in the SCAB during this period do appear to be justified on the basis of this limited data set.

Particulate sampling for this analysis was carried out at eight sites in the SCAB using two high volume samplers at each sampling station in order to provide two identical samples for each experiment, one for "archival" storage and one for immediate analysis. The sampling sites, which were chosen to typify several types of particulate and gaseous pollutant environments, are shown in Figure 1. The locations of the sites are as follows:

No. 1. West Los Angeles, 8 kilometers east of the Santa Monica coastline and several hundred meters west of Freeway I-405.

No. 2. Westwood, east of I-405 and approximately 500 meters west of Westwood Village and UCLA.

No. 3. Long Beach, near the Long Beach industrial area and several freeways.

No. 4. Downtown Los Angeles (California State University).

No. 5. Costa Mesa, 5 kilometers southeast of the Huntington Beach power plant.

No. 6. Claremont, a heavily impacted intermediate smog receptor site.

No. 7. Fontana, two kilometers northeast of a steel mill.

No. 8. Riverside (ARB Mobile Laboratory for Air Pollution Research, located at the University of California), a second smog receptor site.

Samples for this initial geographical and temporal particulate mutagen assay were collected during six consecutive 12-hour periods beginning at 9 p.m. July 11, 1979, on prewashed Teflon-impregnated glass fiber filters (Pallflex TA60A20). Calibration and flow stability of the samplers were checked before and after the collection period and were found to be satisfactory.

The exposed filters were weighed to determine particulate loadings and grouped into three sets of sixteen filters each (representing the "day" and "night" samples from each site). Each set of filters was extracted and the extracts were filtered, reduced in volume in vacuo (>20 torr.), transferred to tared Teflon-sealed vials, and evaporated to constant weight ( $\pm 1\%$ ) with a stream of dry nitrogen. Weighed residues were dissolved in

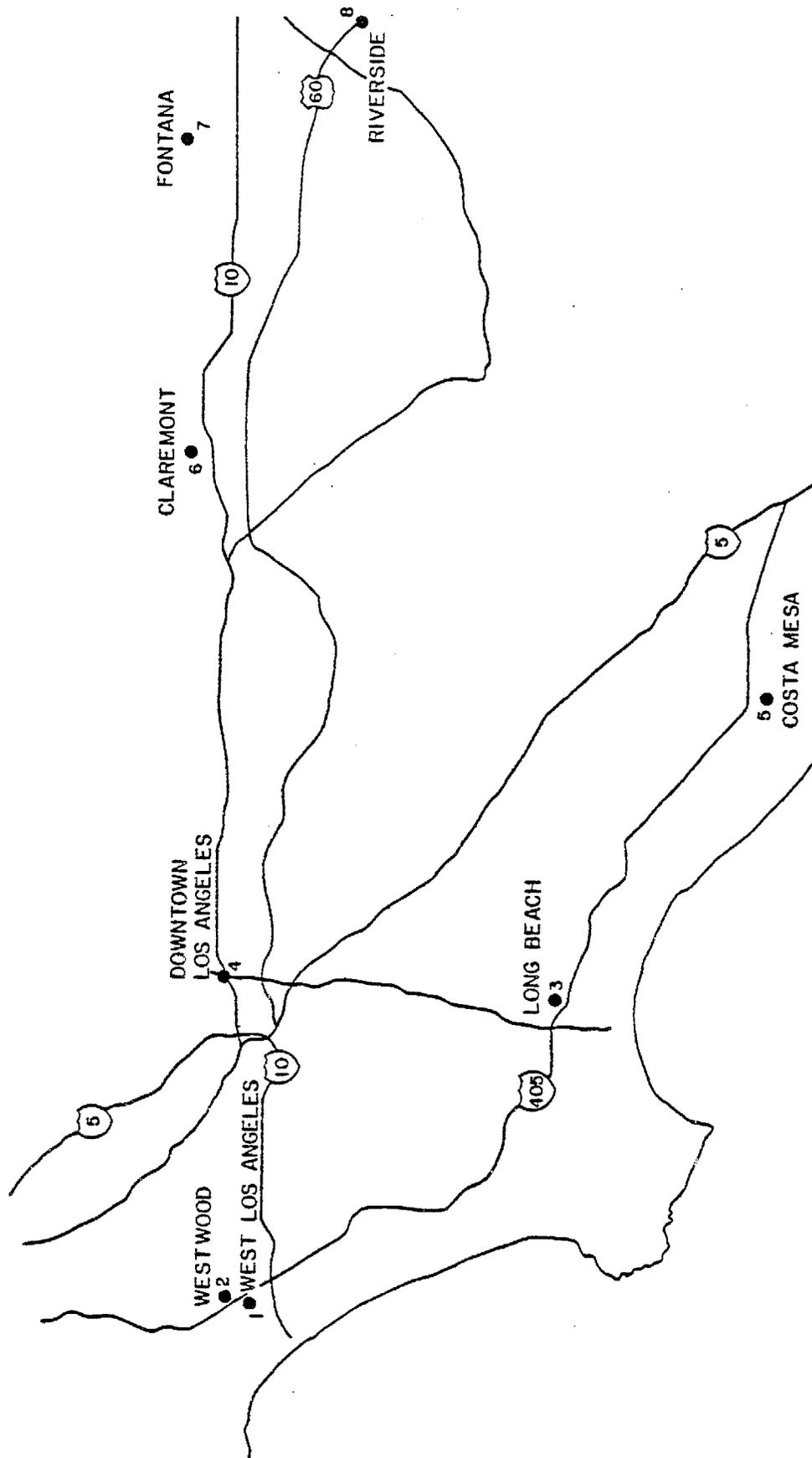


Figure 1. Location of the sampling sites for the geographical study of atmospheric mutagens.

dimethylsulfoxide (DMSO) and submitted for mutagen assay using the improved Ames test protocol (see Section III). Preliminary screens of the samples were performed to determine proper dosage levels (10-400  $\mu\text{g}$ ), the most responsive tester strain (TA98), and appropriate S9 (enzymatic metabolic activation preparation) levels. The quantitative assay of each sample extract and of standard control mutagens was carried out in triplicate, and the linear portion (usually that observed below doses of  $\sim 200$   $\mu\text{g}/\text{plate}$ ) of each set of dose-response data was subjected to a linear least-squares fit.

The specific activities (in revertants/microgram), the mass of the extract, the mass of the collected particulate and the volume of air sampled were used to calculate the quantities referred to in this report as mutagen density (revertants/ $\text{m}^3$ ) and mutagen loading (revertants/mg particulate). (See Section VII for methods used to calculate these quantities.) Mutagen density represents the volume concentration of particulate-borne material mutagenic toward TA98, and ranged from eight to 62 revertants/ $\text{m}^3$  among the sampling sites. Mutagen loading values represent the concentration of material mutagenic toward TA98 on a mass basis, and ranged from 110 to 610 revertants/mg particulate among the sampling sites.

Inspection of our data for this period indicates the following temporal and geographical effects:

- The Costa Mesa (No. 5) and West Los Angeles (No. 1) air exhibited low levels of mutagenic activity which showed insignificant diurnal variation. This would seem to indicate that the Costa Mesa site was not strongly influenced by the nearby power plant during the sampling period. In contrast, the Fontana site (No. 7) near a steel mill apparently received a high mutagen dose, also with no significant diurnal variation.

- The five remaining sites can be divided into two groups based on the observed diurnal variation in measured activity; the Westwood (No. 2), Long Beach (No. 3), and downtown Los Angeles (No. 4) locations experienced a decrease in mutagen density at night, while the Claremont (No. 6) and Riverside (No. 7) measurements revealed the opposite behavior, i.e., a decrease in mutagen density during the day. This may reflect a transport phenomenon, and we have gathered meteorological data to test this hypothesis.

- Comparison of the mutagen loading values for the group of sites showing decreased mutagen density at night (Westwood, Long Beach, and downtown Los Angeles) reveals an interesting difference among the three areas. The Westwood samples can be taken to represent vehicular emissions almost exclusively, as shown by the difference in activity measured on either side of I-405 (sites No. 1 and No. 2). The diurnal behavior of the downtown Los Angeles site (No. 4) mutagen loading figures closely parallels that of the samples taken at Westwood, suggesting that this area is also dominated by vehicular emissions. The Long Beach (site No. 3) values, however, differ substantially from the other two areas in the diurnal variation of mutagen loading. This may be due to differences in meteorology or traffic patterns, or may reflect the impact of nonvehicular (industrial) emission sources in the Long Beach area.

- Among the five sampling sites exhibiting diurnal variation (Westwood, Long Beach, downtown Los Angeles, Claremont, and Riverside), a consistent trend is observable in the ratio of direct to activatable mutagenicity as a function of time of day. This relationship is observable in terms of both mutagen density and mutagen loading in that the ratio of direct to activatable mutagenicity increases at night. We are currently searching for correlations between this phenomenon and the air quality data which have been compiled.

#### G. Ames Mutagenic Assay of Gas-Phase Samples (Section VIII)

We have carried out a preliminary evaluation of three methods of collecting gaseous or vapor-phase materials. The methods investigated were cold trapping of gaseous samples, direct filtered flow of gases over the plated tester strains and the collection and concentration of the sample by use of a solid absorbent (Tenax GC). We have determined that the Tenax GC method can deliver adequate quantities of material for Ames testing when applied to outdoor smog chamber experiments. We are continuing evaluation of this method.

## II. FILTER COMPARISON STUDY: RESULTS OF 1978 EXPERIMENTS

Indirect evidence is accumulating for substantial sampling errors (so-called "filter artifacts") in the analysis of the organic fraction of the particulate collected in polluted atmospheres. These artifacts seem to be due to both physical and chemical processes occurring on the filter. For example:

- Particulate sampling is generally done by high volume filtration, a method which usually assumes that the aerosol has negligible vapor pressure and does not chemically react on the filter material. However, errors due to losses from filters of polycyclic aromatic hydrocarbons (PAH) are known to occur (Brockhaus, 1974; Jones et al., 1976; Pupp et al., 1974; Rondia, 1965; Peters and Seifert, 1980).

- The reactivity of PAH with ambient levels of pollutant gases has been demonstrated (Pitts et al., 1978; Pitts, 1979) and is being further investigated in ongoing experiments at this laboratory. For example, nitration of PAH on glass fiber filters occurs with nitrogen dioxide in air at concentrations as low as 0.25 ppm (the California Air Quality Standard [AQS]). Minute traces of acid vapor such as nitric acid appear to act as a catalyst (Pitts et al., 1978; Pitts, 1979). Additionally, the rapid oxidation of BaP ( $t_{1/2} < 1$  hr) by sub-ppm levels of ozone, as low as the Federal AQS of 0.12 ppm, has recently been demonstrated in this laboratory (Van Cauwenberghe et al., 1980).

- Recently reported research demonstrating the formation of various reactive species, including nitric acid by adsorbed nitrogen dioxide on silica and silica-alumina (Davis and Lunsford, 1978), indicates a possible role for silica-based fibers in mediating the chemical reactions of adsorbed organics.

Our main concern with respect to the mutagenic assay of ambient particulates is that the samples collected be representative of the composition of the airborne atmospheric particulate during the sampling period. Thus, the first set of experiments carried out under this ARB grant were filter artifact studies, designed to determine whether filter surface composition would have an effect on the observed mutagenicity of extracts of the collected particulate.

Experimental. Two sets of collections, extractions, and mutagenic assays comparing five filter types were carried out during the summer of 1978. The first involved simultaneous collection using four filter types: Gelman A-E glass fiber filters, washed and fired at 450 C for five hours (GFF); the same A-E filters, as received from the manufacturer (GFFNW); quartz fiber filters (QAO); and pure Teflon fiber matts (TFF). The second collection was made with two filter types, the washed and fired Gelman A-E (GFF) and Teflon-impregnated glass fiber filters (TIGF).

Ambient particulate was sampled at a site about one-half mile south-east of a steel mill in Fontana, CA. This site was selected to provide a relatively high ratio of primary to secondary emissions, so that oxidative changes in the composition of the filtrate might be more easily assessed. Thus, it was anticipated that samples collected on the most inert of the filter materials would show a larger ratio of activatable (PAH) to direct (PAH reaction products) mutagenicity.

The first set of four filter types was exposed for 102 hours at 40 cfm (total volume: 6,928 m<sup>3</sup>), and the second set of two types was exposed for 81 hours, again at a flow rate of 40 cfm (total volume: 5,502 m<sup>3</sup>). After collection, the filters were equilibrated and weighed to determine particulate loadings. The filters were then extracted by ultrasonication in a 1:1:1 mixture of benzene, dichloromethane, and methanol, and a portion of this "total extract" was submitted to the SAPRC microbiology group for mutagenic testing. The remainder of the extract was reduced in volume and resolved into acid, base, and neutral fractions through an extractive separation using diethyl ether as the organic phase (see Appendix A for details of procedure). These ether-soluble fractions were weighed and then submitted for mutagenic testing. The submitted samples were tested on three of Ames' Salmonella strains, TA98, TA1535 and TA1537, both with and without metabolic activation (S9 levels of 2% and 10% liver homogenate to total mix, v/v). (See Section III for details of procedure.)

Results and Discussion. Table 1 presents the gravimetric data from these experiments as well as the results of the mutagenicity assay with strain TA98.

Table 1. Gravimetric and mutagenic assay data from filter comparison study, Fontana, CA, Summer 1978.

Filter Type:	Collection 1			TFF <sup>d</sup>
	GFF <sup>a</sup>	GFFNW <sup>b</sup>	QAO <sup>c</sup>	
102 hours, 6928 m <sup>3</sup>				
<u>Weight Information</u>				
Collected mass (grams)	1.5264 (31.5)	1.4884 (32.0)	1.5691 (32.6)	1.0962 (34.8)
Total extract (mg)	481.2	476.0	512.0	381.1
Neutrals wt. (mg)	50.8 (3.33)	31.8 (2.14)	28.6 (1.82)	16.6 (1.46)
(% collected mass)	(12.0)	(6.68)	(5.59)	(4.36)
Acids wt. (mg)	57.6 (3.77)	45.9 (3.08)	56.6 (3.61)	32.0 (2.92)
(% collected mass)	(12.0)	(9.64)	(11.1)	(8.40)
Bases wt. (mg)	1.1 (.072)	.85 (0.54)	1.0 (.064)	.55 (.050)
(% collected mass)	(.229)	(.179)	(.195)	(.144)
<u>Ames Reversion Assay with Strain TA98</u>				
	Metabolic Activation *		(-) No, (+) Yes	
TA98	-	+	-	+
Super solvent extract, rev/ $\mu$ g	.17	.3**	.14	.24**
Neutrals, extract, rev/ $\mu$ g	1.3	2.1	2.0	2.5
Acids, extract, rev/ $\mu$ g	.35	.85	2.3	2.3
Rev/sample, neutrals, calc. (x 10 <sup>4</sup> )	6.6	11	5.7	7.0
Rev/sample, acids, calc. (x 10 <sup>4</sup> )	2.1	4.9	13	13
Rev/sample, total extract, calc. (x 10 <sup>4</sup> )	8.0	14	7.0	13
			2.2	2.2
			3.5	3.5
			2.8	3.1

(table continued)

Table 1 (Continued)

Weight Information	Collection 2		TIGF <sup>e</sup>
	81 hours, 5502 m	3	
	GFF <sup>a</sup>		
Collected mass (grams) (% extractable)	1.1351 (26.3)	1.2804 (27.8)	
Total extract (mg)	298.6	356.2	
Neutrals wt. (mg) (% collected mass) (% total extract)	21.1 (1.86) (7.07)	29.6 (2.3) (8.31)	
Acids wt. (mg) (% collected mass) (% total extract)	25.0 (2.20) (8.37)	24.6 (1.9) (6.91)	
Bases wt. (gm) (% collected mass) (% total extract)	27 (.024) (.090)	.46 (.036) (.129)	

## Ames Reversion Assay with Strain TA98

	Metabolic Activation*		
	-	-	+
	No.	(-)	(+) Yes
TA98			
Super solvent extract, rev/ $\mu$ g	50	.19	.39
Neutrals, extract, rev/ $\mu$ g	2.5	2.1	3.1
Acids, extract, rev/ $\mu$ g	4.8	3.3	4.0
Rev/sample, neutrals, calc. (x 10 <sup>4</sup> )	5.3	6.2	9.2
Rev/sample, acids, calc. (x 10 <sup>4</sup> )	12	8.0	9.8
Rev/sample, total extract, calc. (x 10 <sup>4</sup> )	15	6.8	14

- Glass fiber filter, washed and fired (Gelman A-E).
- Glass fiber filter, not washed or fired (Gelman A-E).
- Quartz fiber filter (Pallflex).
- Teflon fiber filter (Zitex).
- Teflon-impregnated glass fiber filter (Pallflex).

\*2% S9 liver homogenate to total mix (V/V).

\*\*Poor dose-response curves.

No clear-cut quantitative conclusions could be drawn from the mutagenicity results because of rather severe departures from linearity in the dose-response curves of some of the fractions. Such poor dose-response curves and variable results led us to study the reproducibility of the Ames assay on a single known direct mutagen, 2-nitrofluorene. Replicate samples of 2-nitrofluorene yielded results varying by as much as a factor of three. For this reason, further ambient collections were postponed until the Ames assay reproducibility could be improved sufficiently to allow more quantitative testing.

It was clear from the gravimetric data (see Table 1), however, that the pure Teflon filters displayed a lower collection efficiency or a lower flow rate than the other types. Also observed was significant variability of mutagenic activity on a weight basis for the same filter (GFF was used in both collections) between the two collection periods although the days were similar in air quality (based on ozone concentrations). For this reason, the field collections for this program were redesigned to be carried out concurrently at all sites sampled. This necessitated lowering the number of sites to eight, and relying on SCAQMD stations for pollutant concentrations.



### III. QUANTIFICATION AND REFINEMENT OF THE AMES SALMONELLA/MAMMALIAN MICROSOME TEST

Our early experiments using the Ames Salmonella/mammalian-microsome mutagenicity test showed a high level of variability in the results (see Section II). This inconsistency was attributable to the Ames test itself, and seriously limited our ability to quantitatively determine the mutagenic activity of environmental samples. We have made an extensive investigation of the factors responsible for the test's poor precision and have developed an improved methodology.

Using the conventional protocol, we obtained results in which the maximum plate counts were as much as 300% that of the minimum plate counts. As shown in Figure 2, our new protocol has resulted in about a one order of magnitude improvement in the plate-to-plate variability (normally, replicate plate counts are within  $\pm 10\%$ ), as well as in greater consistency from experiment to experiment. We are now able to determine more precisely the dose-response relationship for samples of ambient air pollutants, and therefore, to characterize more clearly the relative differences in their mutagenic potency. Such characterization requires that a linear dose-response curve be established; this, in turn, demands sufficient precision to determine a statistically significant linear correlation.

These improvements in precision of the Ames test are the cumulative results of many refinements in the protocol. Over the past several months, we have investigated several potential sources of variability in the Ames test (Belser et al., 1980). In seeking improvements, we have attempted to avoid changes in the basic procedure which would alter its spectrum of sensitivity or otherwise render it incompatible with previous tests. We have found that two factors influence the test's precision and accuracy: physiological factors inherent in the organism itself, and physical effects associated with the manipulation or execution of the test. The two most important innovations arising from our detailed study were:

- The introduction of very careful temperature control during incubation, and
- The development of a method for uniformly distributing the top agar containing the test sample and bacteria over the base agar surface.

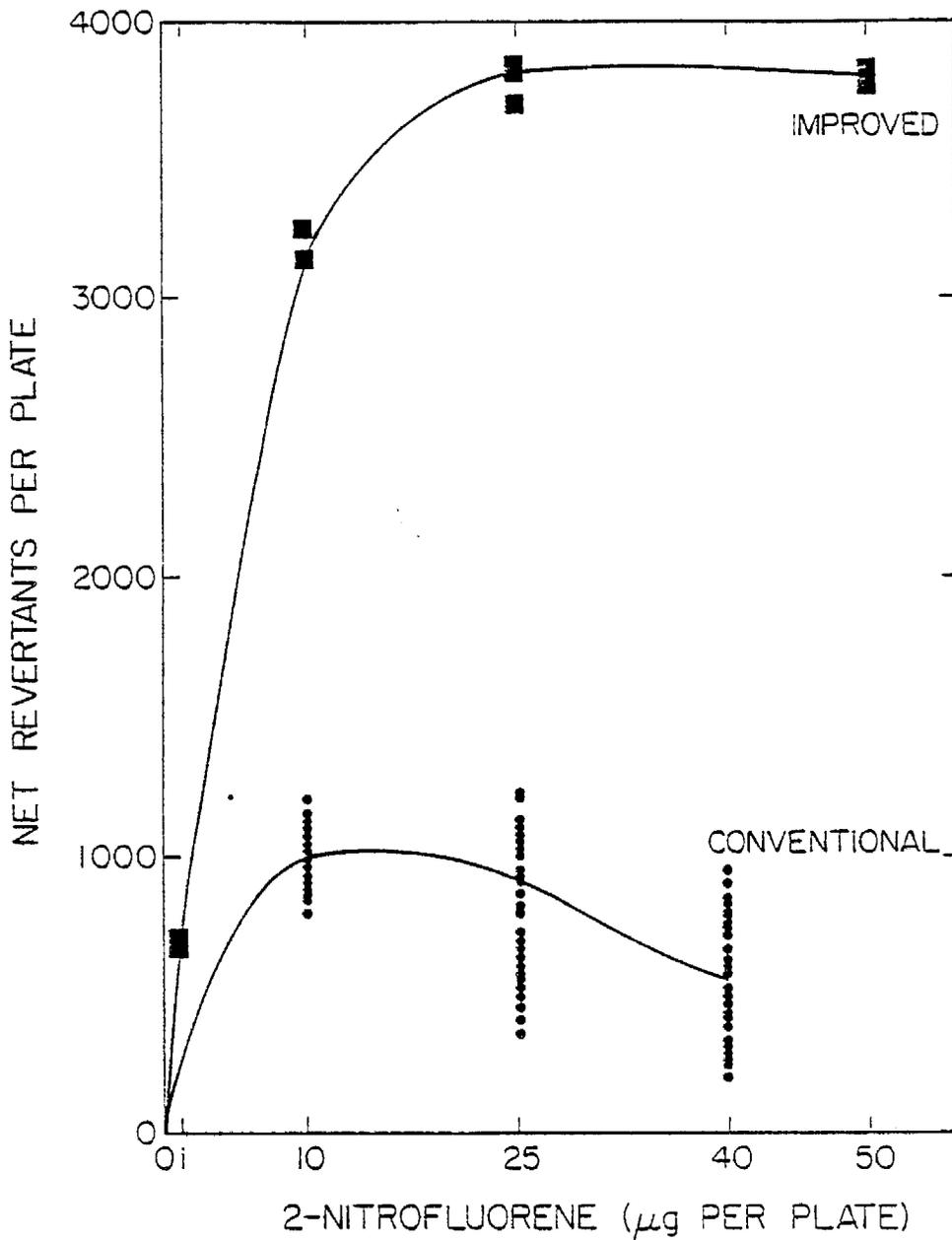


Figure 2. Comparison of the plate-to-plate variability between the conventional Ames *Salmonella*/mammalian-microsome mutagenicity test protocol and the refined SAPRC protocol when assaying 2-nitrofluorene using strain TA98. Fifty replicate plates per dose of mutagen were used in the conventional protocol. The improved protocol used triplicate plates for each dose. The two cultures were comparable, as determined by sensitivity to crystal violet and ampicillin resistance.

Precision between replicate plates was correlated with the distribution of colonies and colony sizes across the plates. The densest areas had very small colonies, whereas other areas contained colonies of an easily counted size. Electronic colony counters seem to be highly sensitive to such variations in colony size; therefore, much of our effort was directed towards reducing this inhomogeneity.

#### Effects Studied.

1) Temperature Effects. We have found that both the temperature and duration of incubation can affect the precision of the Ames assay. More specifically, temperature inhomogeneities in conventional laboratory incubators, whether inherent or induced, may produce nonrandom variability in replicate plates. The incubator in which this effect was first observed was a Thelco Model 6, whose temperature inhomogeneity was determined to be 6°C, side to side and bottom (warmest) to top (coolest). 640 replicate plates (near capacity for this incubator) were prepared by plating strain TA98 with 25 µg 2-nitrofluorene, and incubated in a randomized arrangement for 48 hours in the Thelco. Temperature was monitored at twelve points within the chamber with a Doric DS 350 Type J thermocouple monitor. Following statistical analysis of three separate experiments, the same significant result became apparent: the highest numbers of revertants were found on plates located in the warmest area of the incubator, and the lower counts were found in the coolest. The variation in plate counts was nonrandom and nonsequential ( $F = 45.9$ ,  $\alpha = 0.000$ ), that is, plating order had no effect on the observed number of revertants, whereas differences did correlate with varying temperature. As shown in Figure 3, the observed plate counts (the average for the groups of plates surrounding each of the twelve thermocouple leads) displayed an approximately linear relationship with temperatures below ~35.5°C, and reached a maximum between 35.5 and 37°C. These results are presented only as an indication of the variation in colony counts which may be attributable to local temperature differences within an incubator, and should not be interpreted as representative of the relative number of revertants per plate which would be obtained at different fixed incubation temperatures.

The second incubator examined was a Precision Scientific (Model 805) "B.O.D." box, a modified household refrigerator. During the experiment

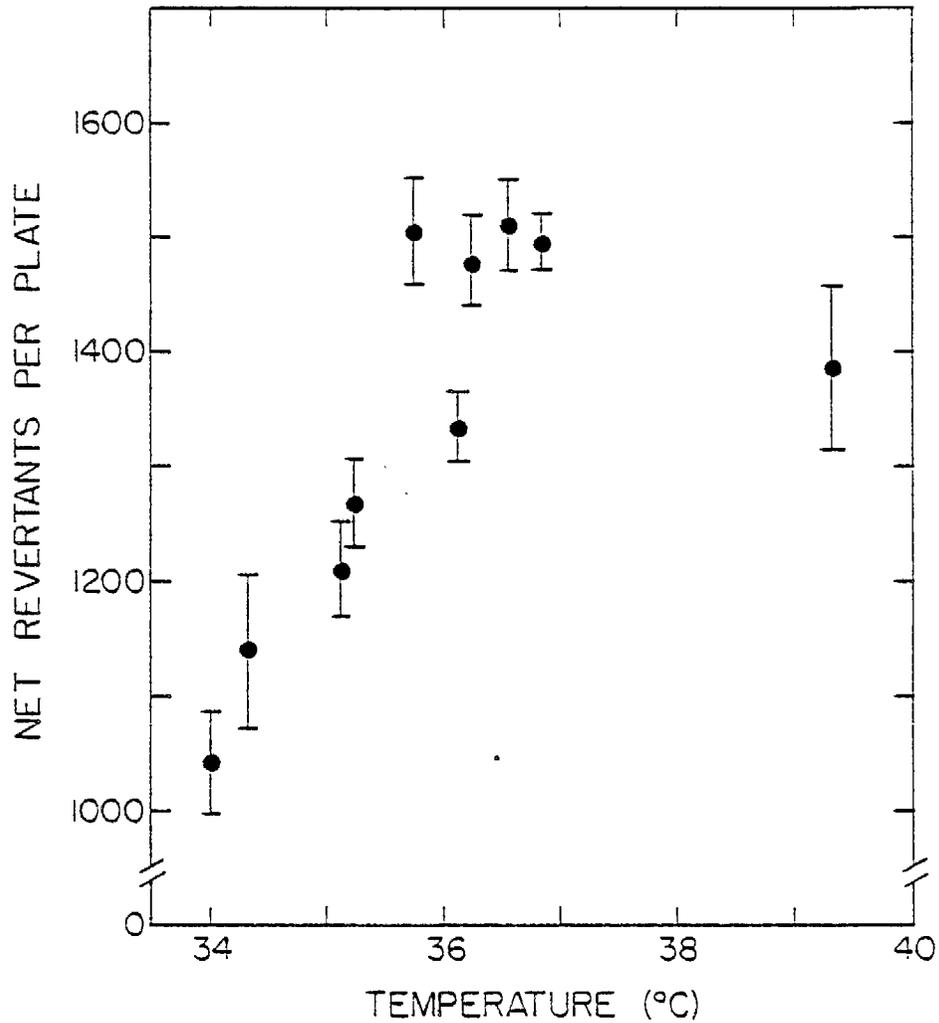


Figure 3. Effect of temperature on revertants per plate using the conventional Ames *Salmonella*/mammalian-microsome mutagenicity test protocol. Net revertants per plate versus temperature after incubation in an incubator having a 6°C maximum temperature gradient in the chamber. Strain TA98 was plated with 25 µg of 2-nitrofluorene. The data represent colony counts in arbitrary groupings of plates around each thermocouple. The error bars represent  $\pm$  one standard error.

temperature was monitored at 12 points in the chamber with a Doric thermo-couple monitor, and a temperature gradient of about three degrees was found. Using the same protocol as described above, nonrandom variation in plate counts was observed which correlated with temperature ( $F = 35.8$ ,  $\alpha = 0.000$ ). When a Forma Scientific Model 3028 CO<sub>2</sub> incubator was tested, a maximum temperature gradient of only 0.25°C was found, and no nonrandom variation in plate counts was observed ( $F = 0.764$ ,  $\alpha = 0.721$ ). However, if this incubator was overloaded, it developed a temperature gradient of 0.8°C, and analyses of variance of the results obtained under these conditions showed statistically significant, although small, differences between shelves ( $F = 21.04$ ,  $\alpha = 0.000$ ).

The question arises as to the cause of this temperature effect. The optimum temperature for growth of most Salmonella species on solid medium is 40°C (Stokes and Bayne, 1957). At 35°C, the growth rate is dramatically lower; thus, a three- or four-degree temperature differential in an incubator may result in colonies growing at different rates on plates placed in different regions of the chamber. This has two implications. First, if the bacteria grow at different rates, the time of exposure of each generation to test mutagens will vary from plate to plate. Each plate contains enough histidine to allow only a few rounds of cell division; hence, although the total number of cell divisions will be the same, the growth time will vary depending upon the temperature. If the mutagen has a short half-life in agar, some plates will have shorter effective exposure times relative to the number of cell divisions. These plate counts may be spuriously low after 48 hours of incubation because some revertant colonies will not be large enough to be accurately counted. This is of particular importance on plates with densely aggregated colonies (1000 or more per plate).

Similarly, the length of time that test plates are incubated might be expected to affect the observed number of revertants. We have found that 63 hours is close to optimum for strain TA98 with 2-nitrofluorene as the test mutagen (Figure 4). Longer incubation produced little or no increase in countable colonies, and some plate drying was apparent (as evidenced by the agar cracking and pulling away from the plate) by

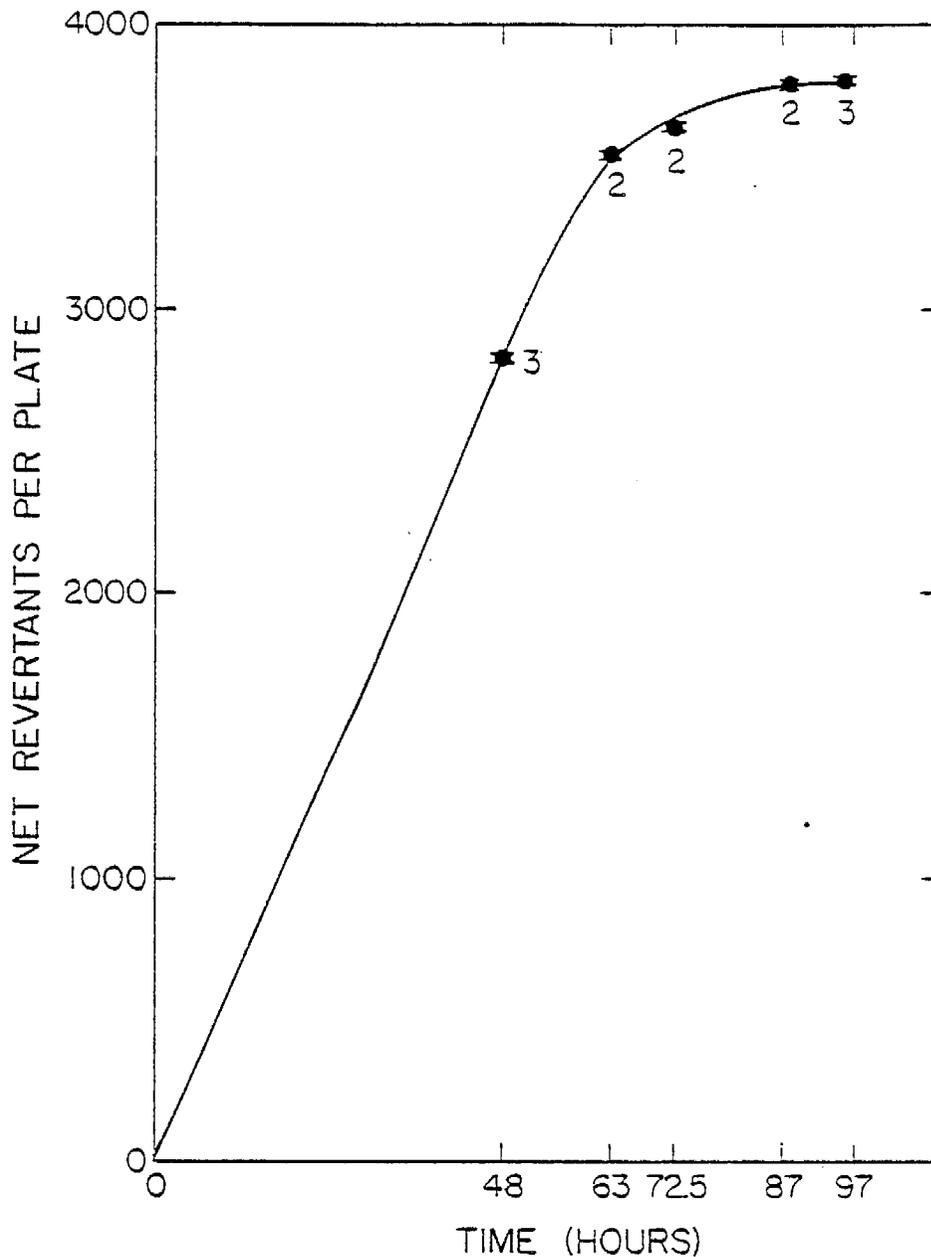


Figure 4. Effect of time of incubation on colony counts using the refined SAPRC protocol. Fifty replicate plates were prepared with 25  $\mu$ g of 2-nitrofluorene and strain TA98. The plates were removed from the incubator (Forma Scientific model 3028) at the indicated times and counted, and then were returned to the incubator. The data were not corrected for nonlinearity of the colony counter at high counts. The error bars represent  $\pm$  one standard error for each determination. The coefficient of variation (i.e., percent standard deviation) is indicated below each symbol.

96 hours. The optimum incubation for other strains and mutagens may be different, depending in a complex way on the toxicity of the compound under test, the time at which the reverse mutations occur, and crowding and mutual growth inhibition of revertant colonies. For quantitatively comparable results, plates should be incubated long enough for the optimum plateau region to be reached. This may be determined by counting plates at twelve-hour intervals and returning them to the incubator between countings.

Initially, an increase in incubation time from 48 to 63 hours resulted in agar drying, which interfered with experimental results. To alleviate this relative humidity (RH) was increased to 80%. Humidity lower than 80% caused drying of the plates, while RH greater than 90% resulted in excessive condensation on the agar surface and spreading of the revertant colonies. We use 80% RH, which is light enough to retard drying of the agar without creating excess condensation. Therefore, we have adopted the use of Forma Scientific Model 3028 forced-draft CO<sub>2</sub> incubators with tightly controlled chamber temperature and relative humidity (RH).

In the Ames test procedure temperature may also be important in the holding of cells in soft agar prior to plating. The bacteria may be exposed to elevated temperature for periods ranging from a few seconds to several minutes. We tested survival of strain TA98 at 45, 47, 49 and 51°C for periods of time up to 120 seconds after the addition of cells. The study was done in triplicate plates and the experiments performed twice. Figure 5 shows the survival curve. At temperatures up to 51°C and times below two minutes, no conspicuous loss of viability occurred. Response to a standard mutagen was also unaffected at temperatures up to 65°C under normal test conditions when the soft agar was plated immediately after addition of cells. Therefore, we conclude that soft agar temperature is not critical, but that cells should not be held at these temperatures for extended periods.

2) Uniform Spreading of Top Agar. When working with mutagen concentrations high enough to induce as many as 1000 revertants per plates, uneven colony size distribution can adversely affect counting accuracy (Brusick, 1978). The colony density becomes so great in some areas of the plate that growth is inhibited, resulting in microcolonies too small to be

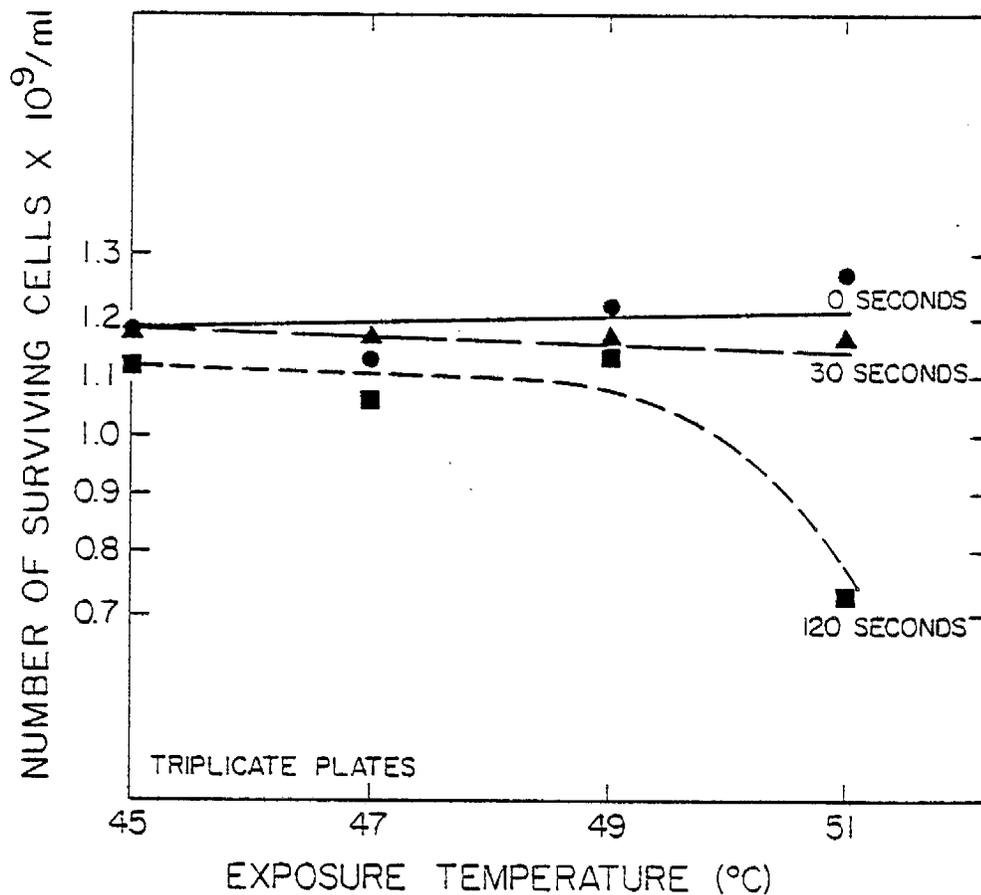


Figure 5. Cell death as a function of time and holding temperature. Aliquots of diluted culture of strain TA98 were added to tubes containing L broth top agar. The tubes were equilibrated to the indicated temperatures in a water bath. At the indicated times, tubes were withdrawn from the water bath and plated on rich medium (L broth) to determine viable count.

counted by electronic colony counters. We attributed this regional variability and uneven colony size distribution to nonuniform spreading of the viscous top agar over the base agar surface.

We have examined a number of ways of improving top agar uniformity, including increasing the volume of soft agar used per plate, altering the soft agar concentration, and implementing mechanical methods to spread the soft agar coat. For example, we have found that incomplete mixing of top agar, bacteria, and mutagen can lead to increased variability in replicate plate counts. The recommended mixing technique [rolling the tube between one's hands prior to pouring (Ames et al., 1975)] does not result in complete mixing. Flicking the tube with a finger several times, a practice widely used in microbiology, results in more complete mixing. However, mechanical vortex mixers work best, if care is taken to avoid formation of bubbles in the soft agar. The bubbles are not easily broken up and can be registered as colonies by electronic colony counters. The most promising approach was the use of a level table with built-in vibrators upon which to place the freshly poured plate for solidification. We have designed and built such an agar leveler using five modified loudspeakers as vibrators, plus an oscillator and amplifier to drive them (Figure 6). The freshly poured plate is placed on the vibrating platform which levels the top agar layer during solidification. Using this machine, we were consistently able to obtain plates with evenly distributed colonies. Also, there was a corresponding improvement in plate-to-plate variability to within  $\pm 10\%$  or better. The data shown in Table 2 were taken from an experiment in which tubes were either flicked or vortexed and the top agar leveled either by vibration or swirling. The efficacy of each technique was assessed, both by calculating the standard error and by determining the number of plates with nonuniform colony distributions. The major improvement was clearly produced by vibration, but machine vortex mixing has also yielded a consistent improvement in plate-to-plate variability.

3) Effect of the Number of Cells Plated. We have conducted experiments to test the effect of the number of cells plated on the number of revertants per plate for standard mutagens. Overnight cultures of each of the five strains were grown to approximately the same turbidities. Titers were determined by dilution and plate count (in our experience, overnight

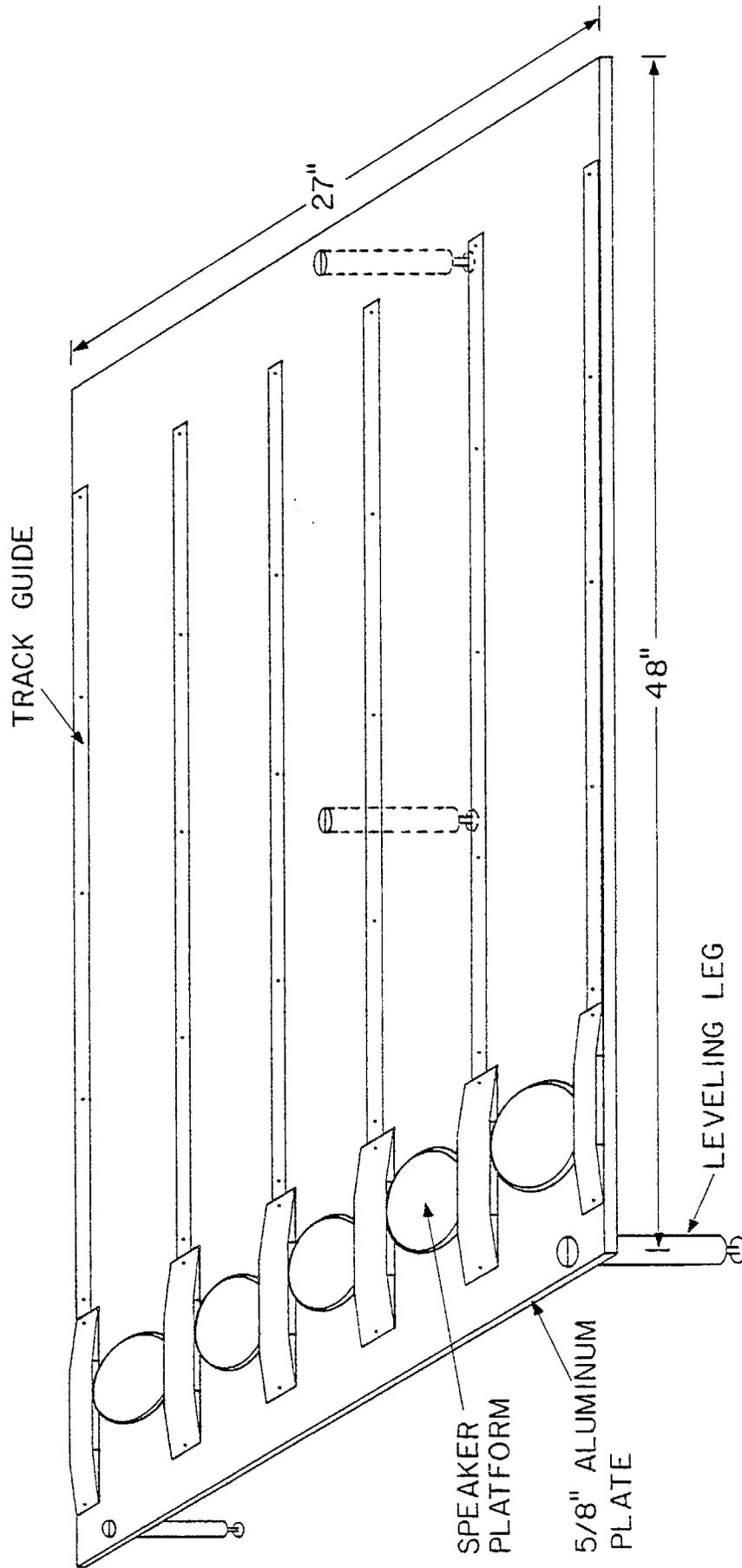


Figure 6. The oscillator/vibrator agar leveler (OVAL).

titers are somewhat variable from day to day). Each culture was tested undiluted and at 1:2, 1:4, 1:8 and 1:16 dilutions. The undiluted culture was tested at 0.2 and 0.1 ml per plate and the diluted cultures at 0.1 ml per plate. As shown in Figure 7, the highest number of revertants were obtained at  $1 \times 10^8$  cells per plate, with slightly fewer at  $5 \times 10^7$  cells per plate. Over a range from  $5 \times 10^7$  cells down to  $6 \times 10^6$  cells per plate, the number of revertants were somewhat lower than those at  $1 \times 10^8$  cells per plate, but with no significant variations within this range. Thus, the relationship between number of cells plated and number of revertants per plate is not linear, and the actual number of cells plated is not critical. However, we recommend that overnight titers be adjusted to yield inocula of about  $1 \times 10^8$  cells, thus avoiding even the small amount of variability which can be ascribed to inconsistency in the number of cells plated.

4) Effect of Base Agar Volume. Another variable in the experimental protocol is base agar volume in the Petri plates. In a sample of unknown composition, some compounds are likely to be water-soluble. Diffusion of these water-soluble compounds into variable volumes of agar in the base layer could produce inconsistencies in the dose of mutagen received by the cells in the top layer. Figure 8 illustrates this point. Two mutagens, one diffusible (2-nitrofluorene) and one relatively nondiffusible [benzo(a)pyrene] were tested on plates containing

Table 2. Effect of mixing techniques on nonuniform distribution of colonies in the Ames Salmonella/mammalian-microsome mutagenicity test.

	FLICKED		VORTEXED	
	% NONUNIFORM PLATES	STANDARD ERROR/MEAN	% NONUNIFORM PLATES	STANDARD ERROR/MEAN
Not vibrated	84.6	0.022	79.4	0.025
Vibrated	22.9	0.007	7.3	0.006

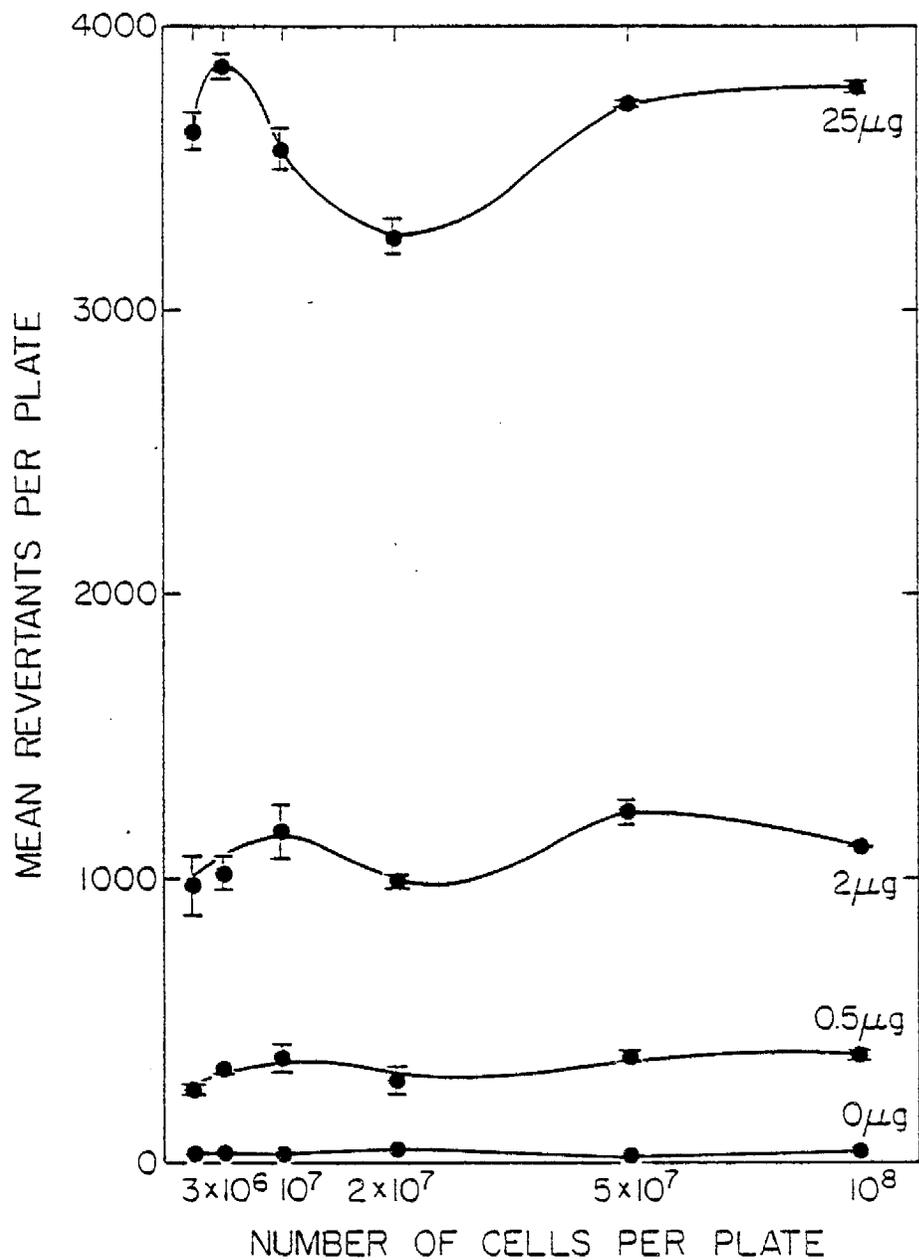


Figure 7. The effect of the number of tester cells added per plate for three different doses of 2-nitrofluorene. The plates were prepared with dilutions of an overnight culture and incubated 63 hours in the Forma Scientific incubator. Three replicates were plated for each titer and each concentration of mutagen. The data shown are for strain TA98, and the error bars indicate  $\pm$  one standard error.

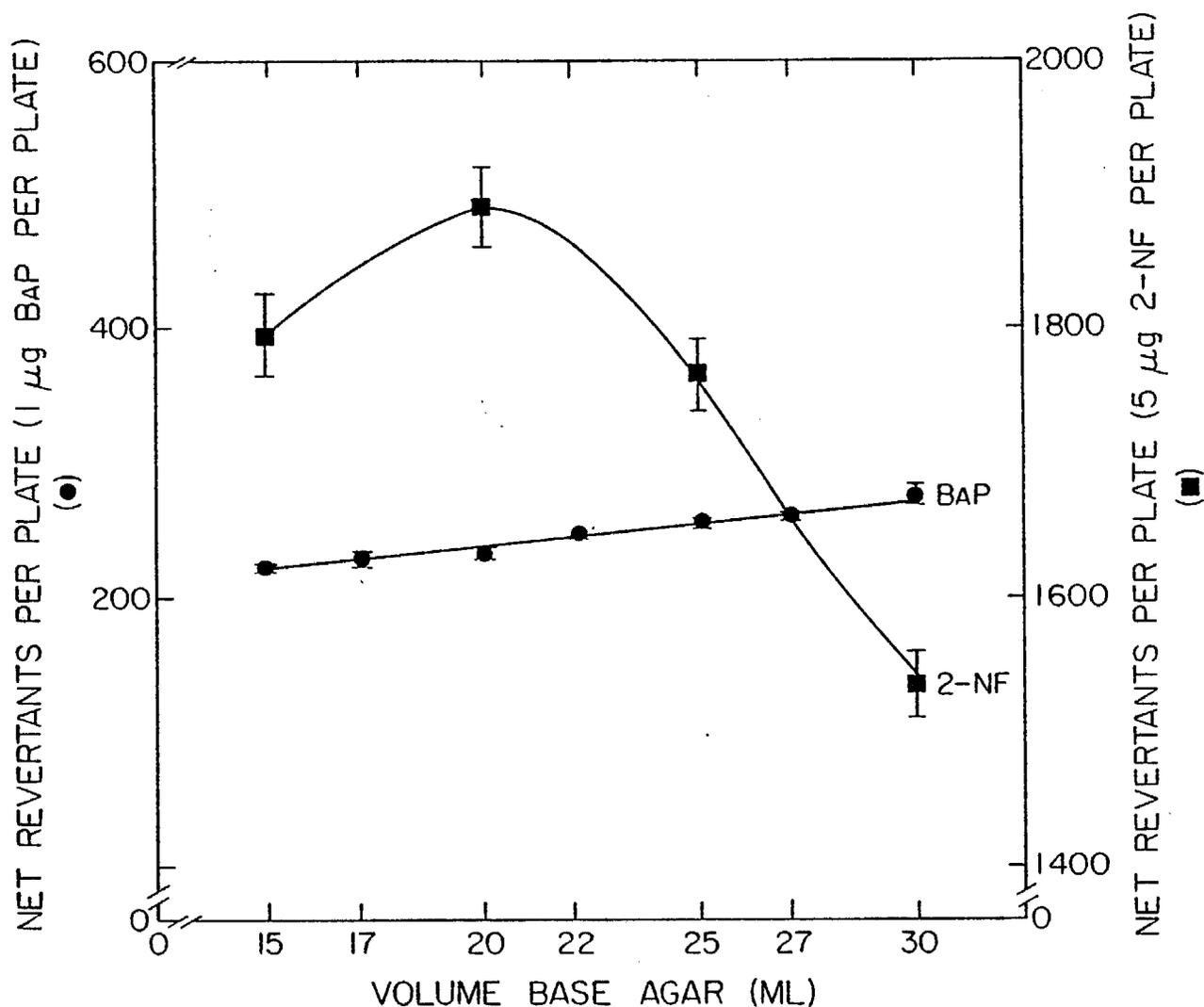


Figure 8. The mean number of revertants of strain TA98 per plate versus base agar volume for a diffusible and nondiffusible mutagen. The base agar was Vogel and Bonner minimal medium supplemented with 0.1 mM CaCl<sub>2</sub>, plus 2% W/V D-glucose and 1 μg/ml biotin. Plates were poured using a Manostat automatic plate pourer which dispersed volumes accurate to better than ±5%. Benzo(a)pyrene (BaP) [●] (1 μg/plate) was activated using 5% (v/v) S9 liver homogenate and is an example of a relatively nondiffusible mutagen. The diffusible mutagen was 2-nitrofluorene (2NF) [■] and the dose was 5 μg/plate. Error bars indicate ± one standard error.

various volumes of the base agar. The average number of revertants per plate was inversely related to base agar volume for the diffusible mutagen, but increased slightly with agar volume for the benzo(a)pyrene. The latter effect may have been due to increased availability of nutrients in the high agar volume plates or, perhaps, to the downward diffusion of some toxic substance. Some of the low volume plates appeared to be drying out during incubation and we view this as introducing another potential problem in quantitation of results. This drying effect may have been responsible for the decreased number of revertants observed on the 15 ml 2-nitrofluorene plates. We conclude that 20 ml plates are best, and that these should be machine poured to assure volume uniformity.

5) Automated Colony Counter Calibration Effects. To provide consistent and accurate plate counts, we are utilizing a New Brunswick Biotran II colony counter. We have found that periodic lens focusing and adjustments of the F-stop of the colony counter are necessary to ensure proper counting sensitivity. Furthermore, electronic plate counts should also be corrected to a curve-fitting formula derived regularly from a comparison of hand and machine-counted plates. We have found, as have others (Brusick, 1978; Chriswell et al., 1978; Epler et al., 1978; New Brunswick Scientific Co., 1979), that the relationship between actual and machine-counted colonies is not linear at higher colony density (> 100 per plate). Moreover, the variability in plate counts also increases with colony density. This may be attributable to machine characteristics.

6) S9 Suppression and Optimal Concentrations. In a number of our experiments, we have observed, as have others (De Flora, 1978), the suppression of frequency of revertants when S9 was added. Therefore, we have attempted to further characterize the effect of S9 concentration on reversion frequency. Two different activatable mutagens, 2-aminofluorene and BaP, were tested at two sample concentrations with five levels of S9 using TA98 as the tester strain. The results of this experiment are shown in Figures 9 and 10. The data for the low concentration of BaP (Figure 9) show that optimum activation occurs at 2% (v/v) of the liver homogenate in the total S9 mix, and that increasing concentrations of S9 suppress the number of revertants. At the higher concentration of BaP, optimum activation occurred at 8% (v/v) S9 per plate, with suppression at higher concentration. It is

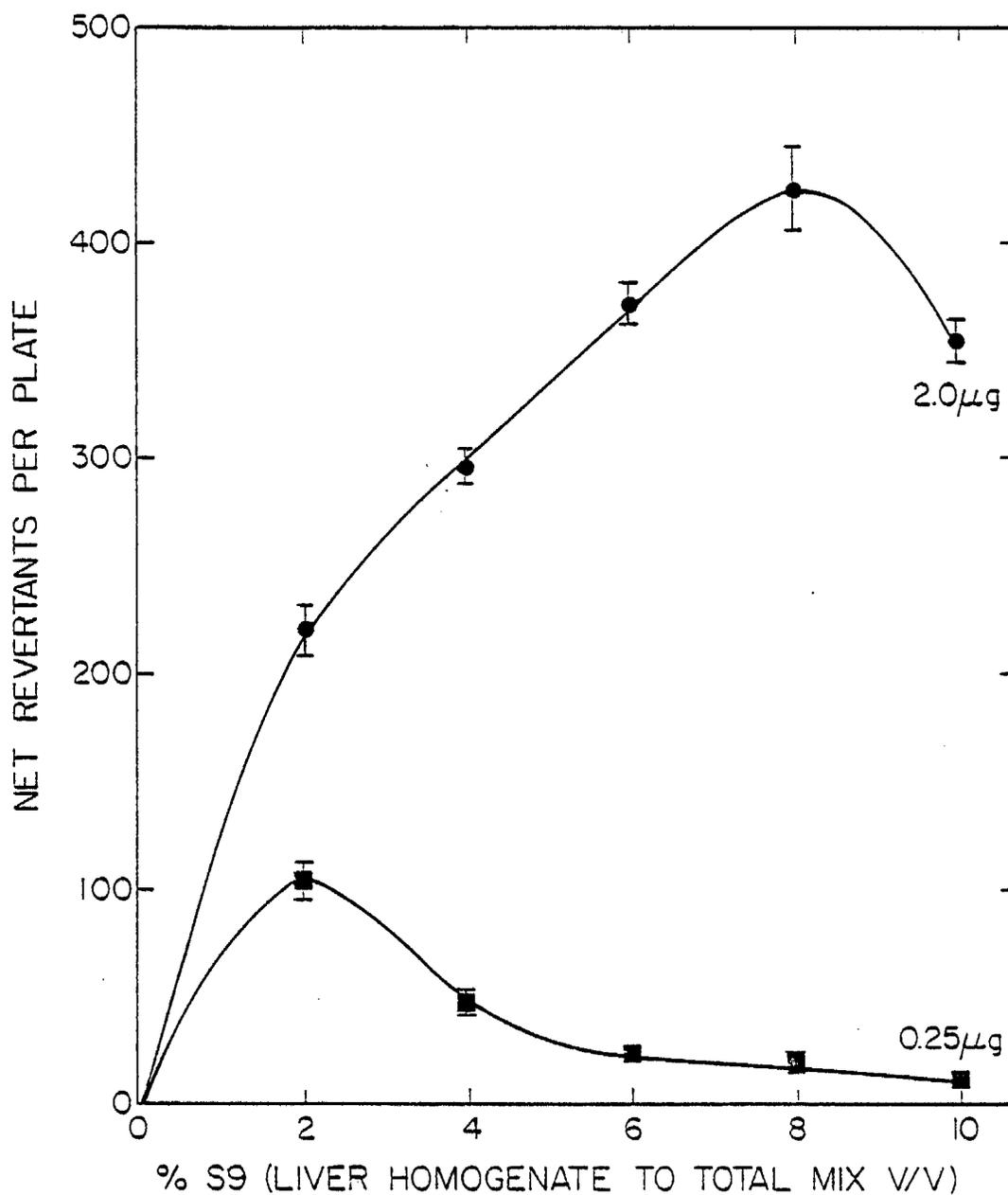


Figure 9. Net revertants per plate of strain TA98 versus S9 concentrations for benzo(a)pyrene at two dose levels. Note that the optimum S9 concentration depends on the amount of mutagen. Triplicate plates were prepared for the experiment; the error bars represent  $\pm$  one standard error.

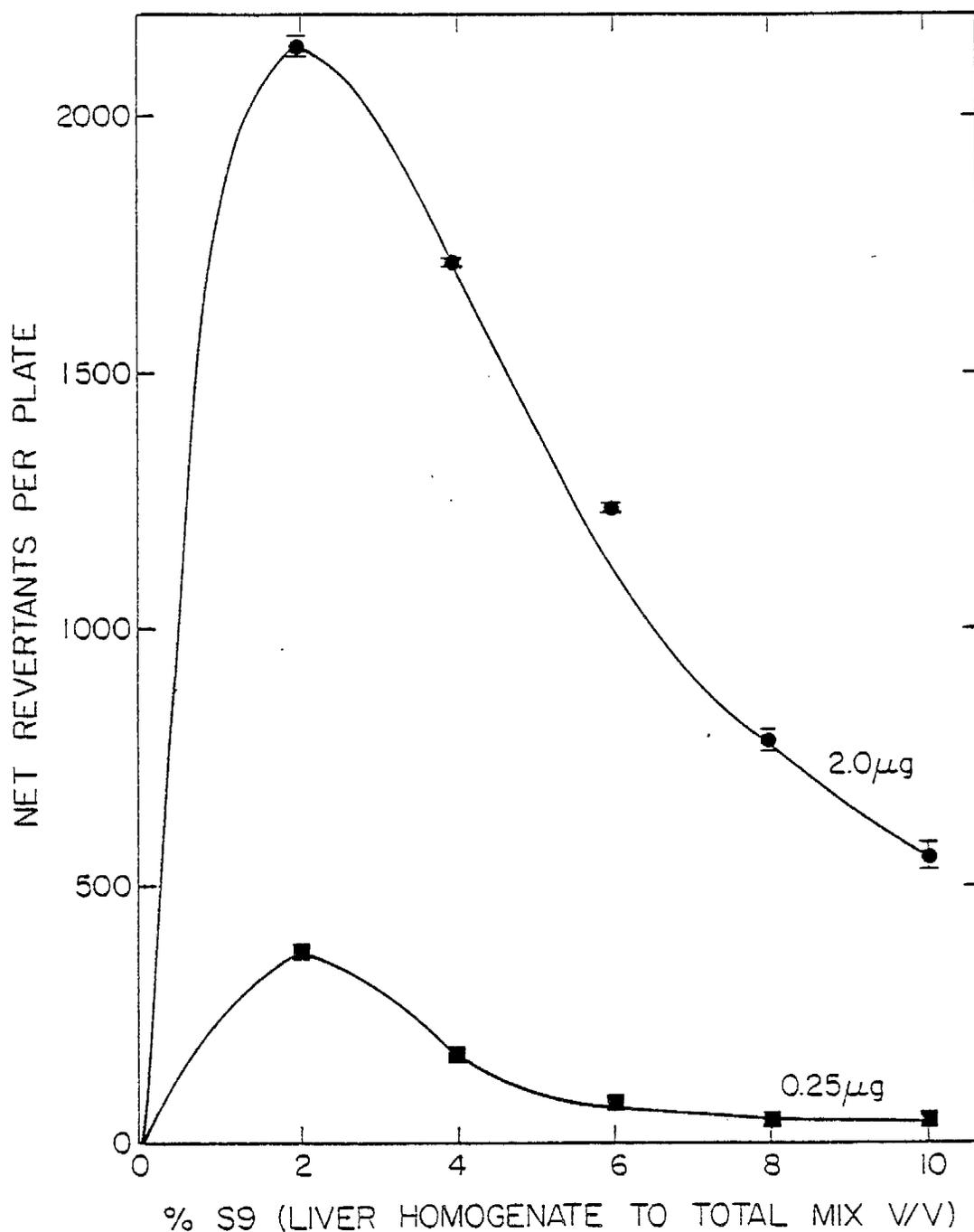


Figure 10. Net revertants per plate of strain TA98 versus S9 concentrations for 2-nitrofluorene at two dose levels. Note the optimum S9 concentration is independent of the amount of mutagen. Triplicate plates were prepared for the experiment; the error bars represent  $\pm$  one standard error.

interesting that at both the high and low concentrations of 2-aminofluorene (Figure 10), the optimum S9 concentrations for activation were identical at 2% (v/v) S9 per plate.

We recommend the use of three S9 concentrations for screening samples of unknown mutagenicity: a "low" (2% v/v), a "medium" (10% v/v) and a "high" (up to 40% v/v) concentration. Thus, if a mutagen of the 2-aminofluorene type is present, it will not be suppressed by high S9 and yield a false negative, whereas, if a mutagen such as BaP is present, a more accurate estimate of the effective S9 concentration range for further quantitation will be obtained. In recent experiments, we have confirmed earlier observations by others (Dickson, 1978; Eisenstadt, 1978) that perylene is an activatable mutagen only at very high S9 concentrations. The optimum S9 concentration is about 40% (v/v) on strain TA1537. Further, the efficacy of each batch of S9 should be carefully established for quantitative determinations of activatable mutagenic activity using a standard mutagen, before results obtained using different batches can be considered comparable.

#### 7) Application of the Ames Test to Screening Environmental POM.

Our usual test materials are complex mixtures of chemicals which may contain many cytotoxic compounds as well as mutagens. Because of these properties of ambient air pollution samples, we have rejected the use of spot tests as a preliminary screening procedure for two reasons. First, many mutagens may be nondiffusible and will show no activity in a spot test. Second, if a diffusible cytotoxic compound is present, it might inhibit the growth of revertant colonies in the vicinity of the spot. Both effects could lead to falsely negative test results. [An improved screening method has recently been reported which appears to be somewhat more quantitative than spot tests, while additionally avoiding its inherent disadvantages (McMahon et al., 1979).]

These considerations led us to develop a preliminary screening test based upon the agar layer method. For this, we use all five tester strains. Each sample is tested at three concentrations over a thousand-fold range, and two levels of S9 are used for activation. We prepare duplicate plates for each test. We do not expect quantitative conclusions from these tests, but they do establish the optimum ranges of sample and S9

concentrations, and enable us to select the most responsive strain or strains for quantitative testing.

For quantification of sample mutagenicity, we use the most sensitive strain(s), select the optimum S9 concentration range and test as many concentrations of the sample as sample size permits to best characterize the linear region of the dose response curve. These concentrations are chosen to cover at least one order of magnitude around the optimum as determined from the preliminary test.

Each day that samples are tested, a full battery of controls is run. It is essential that the strains be routinely checked for the presence of the plasmid and the correct genotype. The plasmid-carrying strains (TA98 and TA100) respond differently to some mutagens than their respective parental strains (TA1538 and TA1535). For example, TA100 responds to both base substitution and frameshift mutagens, whereas TA1535 responds only to base substitution mutagens. The plasmid can be easily lost; single colony clones should be isolated and tested frequently to ensure the genotype of each strain. In each experiment, spontaneous reversion frequencies are also determined. Further, it is desirable that the mutant strains be tested against known mutagen standards at several doses as an internal control of their response. The data base derived from repeating dose-response determinations of known mutagens may allow quantitative comparisons from experiment to experiment. After a standard response is established, variations from this response can then be used to adjust test data appropriately. The control compounds which we use are:

- (i) sodium azide -- strain TA1535, TA100
- (ii) 9-aminoacridine -- strain TA1537
- (iii) 2-nitrofluorene -- strains TA1538, TA98, TA100
- (iv) benzo(a)pyrene (with activation) -- strains TA1537, TA1538, TA98, TA100
- (v) 2-aminoanthracene (with activation) -- strains TA1535, TA1537, TA1538, TA98, TA100

We are exploring the use of oxygenates of common ambient polycyclic aromatic hydrocarbons such as the hydroxy-BaP isomers. We hope to develop standards closely resembling some species we believe to be formed in atmospheric transformations of pollutants.

Certain other precautions are desirable in the handling and treatment of unknown test chemicals. The use of inactinic red light in the laboratory reduces the photodecomposition of unstable materials. We formerly used a "black light" to detect fluorescent spots on TLC (thin layer chromatography) plates which had been used to resolve mixtures of mutagenic compounds. Use of the black light was discontinued and red lights were installed in the laboratory after one of the fluorescent spots disappeared within a few seconds. We have also determined that known mutagens such as benzo(a)pyrene can be oxidized in the presence of actinic light. Solutions of standard test mutagens should be made up just prior to their use. We are investigating storage procedures for individual aliquots from the same stock solution.

As noted above, the improvements and modifications described here have resulted in about one order of magnitude improvement in the precision of our Ames tests. Formerly, plate-to-plate variation in replicate plates was as much as 300%. In our opinion, these modifications render the Ames method sufficiently quantitative so that test compounds can be accurately characterized as to mutagenic potency. Such characterization requires that a linear dose-response curve be established, and this, in turn, demands sufficient precision to determine a straight-line relationship.

There are a number of areas in which further improvements to the Ames method might be found. For example, it might be possible to improve the sensitivity of the test by inclusion of enhancing compounds, such as adenine (Fisch and Beck, 1978), in the growth medium. Nonmammalian activating systems, such as plant extracts, might be advantageous in assessing the toxicological fate of environmental chemicals in the human food chain.



#### IV. FILTER COMPARISON STUDY: RESULTS OF 1979 EXPERIMENTS AND SELECTION OF FILTER MEDIA

The interference due to a "filter artifact" in the collection of ambient particulate could be due to a number of physical and chemical processes associated with the filtration technique. Those which are expected to result from properties of the filter material include adsorption of gas-phase organics onto the filter surface and the catalytic activity of that surface with respect to reactions between reactive gases and the collected material. The effects of these processes upon the measured mutagenicity of the extracted organic material are impossible to predict, since such reactions may lead either to destruction of mutagens initially present or to the creation of mutagens from nonmutagenic material (Pitts, 1979; Pitts et al., 1978; Tomingas, 1979). Thus, an attempt to assess the importance of the contribution of surface activity to the measured mutagenicity by a simple comparison of dose-response relationships of the extract may be complicated by this ambiguity.

Experimental. We have concluded the collection, extraction, and fractionation of three sets of samples obtained in filter comparison studies during the spring of 1979. Each set involved a simultaneous collection of samples on four filter types:

- QAO - Pallflex Q2500 quartz fiber filters, heated to 450°C for five hours.
- EPA - Schleicher and Schuell borosilicate glass fiber filters recommended by the EPA in 1979, used as received.
- GFF - Gelman A-E glass fiber filters, washed sequentially with methanol, dichloromethane, and toluene, vacuum dried, and heated to 450°C for five hours in an annealing oven.
- TIGF - Pallflex T60A20 Teflon-impregnated glass fiber filters, extracted with methanol and cyclohexane and air-dried.

Ambient particulate sampling was performed using 16 calibrated high-volume samplers located on the roof of the Physical Sciences building at Harvey Mudd College in Claremont, CA. Four samplers were devoted to each filter type. The exposed filters were equilibrated at 75°F, 50% RH for 12

hours and weighed to determine TSP loadings; the four filters of each type were grouped together and treated as a single sample. Each group of filters was extracted by ultrasonic agitation with a 1:1:1 mixture of methanol, dichloromethane, and toluene, and the extracts were reduced in volume in vacuo (>20 torr) and subjected to an extractive acid/base/neutral fractionation using diethyl ether as the organic phase (Hueper et al., 1962). The base used for isolation of "acidic" components of the extracted particulate organic matter differed in the two fractionation methods employed. The first collected sample was processed using 2N NaOH while the second and third sample set treatments employed 10% KHCO<sub>3</sub>.

The ether-soluble acid, base, and neutral fractions obtained from this procedure were transferred to tared, Teflon-seal vials and evaporated to constant weight (+10%) with a stream of dry nitrogen. The gravimetric data obtained from these procedures are presented in Table 3.

The acidic fractions and a portion of each neutral fraction were dissolved in DMSO, frozen in liquid N<sub>2</sub>, and delivered to the microbiology group. Quantitative screens were performed on each sample set with strain TA98. A 2% (volume liver to volume mix) S9 concentration was employed to screen for activation. A sodium phosphate buffer (0.5 ml, 0.0667 M) was added to non-S9 samples to maintain volume consistency with the S9 samples. The acid and neutral samples were screened in triplicate at concentrations of 10, 20, 40, 60, 80, 100, 200 and 400 µg of sample per plate. The basic fractions could not be quantitatively screened due to their small sample size. A linear regression was performed on the linear regions with the slope determined in revertants per microgram for each sample. The results of this assay are presented (in terms of specific activity, revertants/µg) in Figures 11-13.

A portion of each neutral fraction was subjected to reversed-phase high performance liquid chromatography, and the resulting "fingerprint" chromatograms were inspected for qualitative and quantitative variations in the UV-absorbing components which might reflect the existence of an artifact induced by filter surface characteristics.

Table 3. Gravimetric data from filter comparison study, Claremont, CA,  
Spring 1979.

Experiment #1. Old Protocol Used for Extraction and Separation

5/14/79: Exact times not known due to malfunction; approximate  
average 61 hr per filter, four filters of each type

	Acids		Bases		Neutrals		Total ESO	ESO <sub>x100</sub>
	mg	(% Total)	mg	(% Total)	mg	(% Total)	mg (100%)	TSP
QAO	39.062	(43.4)	5.551	(6.2)	45.353	(50.4)	89.966	6.1%
EPA	49.967	(44.1)	4.149	(3.8)	59.292	(52.3)	113.408	5.5%
GFF	50.717	(45.3)	4.563	(4.1)	56.558	(50.6)	111.838	5.6%
TIFG	51.512	(42.5)	4.358	(3.6)	65.323	(53.9)	121.193	6.2%

Experiment #2. New Modified Protocol Used for Extraction and Separation

6/1-4/79: 74 hr per filter, four filters of each type

	Acids		Bases		Neutrals		Total ESO	ESO <sub>x100</sub>
	mg	(% Total)	mg	(% Total)	mg	(% Total)	mg (100%)	TSP
QAO	69.731	(47.6)	2.786	(1.9)	74.052	(50.5)	146.569	6.6%
EPA	71.092	(47.9)	2.392	(1.6)	74.925	(50.5)	148.409	6.2%
GFF	77.394	(48.8)	2.536	(1.6)	78.745	(49.6)	158.675	7.0%
TIGF	79.852	(47.1)	2.987	(1.8)	86.617	(51.1)	169.456	8.0%

Experiment #3. New Modified Protocol Used for Extraction and Separation

5/23-25/79: 45 hr per filter, four filters of each type

	Acids		Bases		Neutrals		Total ESO	ESO <sub>x100</sub>
	mg	(% Total)	mg	(% Total)	mg	(% Total)	mg (100%)	TSP
QAO	51.686	(50.1)	2.362	(2.3)	49.112	(47.6)	103.160	7.3%
EPA	56.258	(53.8)	1.680	(1.6)	46.697	(44.6)	104.635	6.8%
GFF	42.242	(46.6)	1.587	(1.8)	46.870	(51.6)	90.699	6.1%
TIGF	52.861	(50.2)	2.080	(2.0)	50.406	(47.8)	105.347	7.5%

ESO = ether soluble organics, TSP = total particulate weight collected.

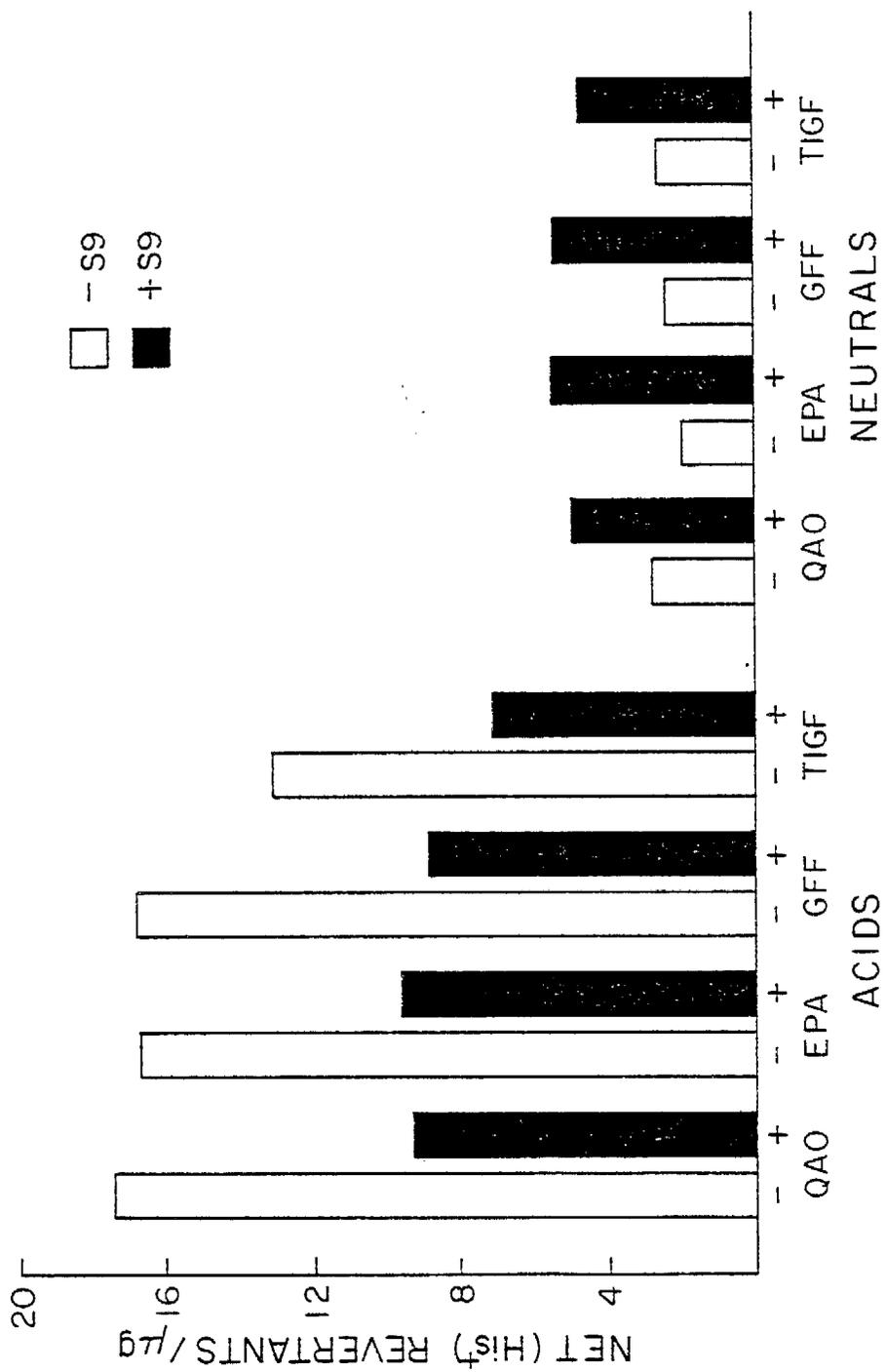


Figure 11. Specific mutagenic activity (revertants/ $\mu$ g) on strain TA98 of filter comparison study samples. Set 1, Claremont, CA, May 14, 1979.

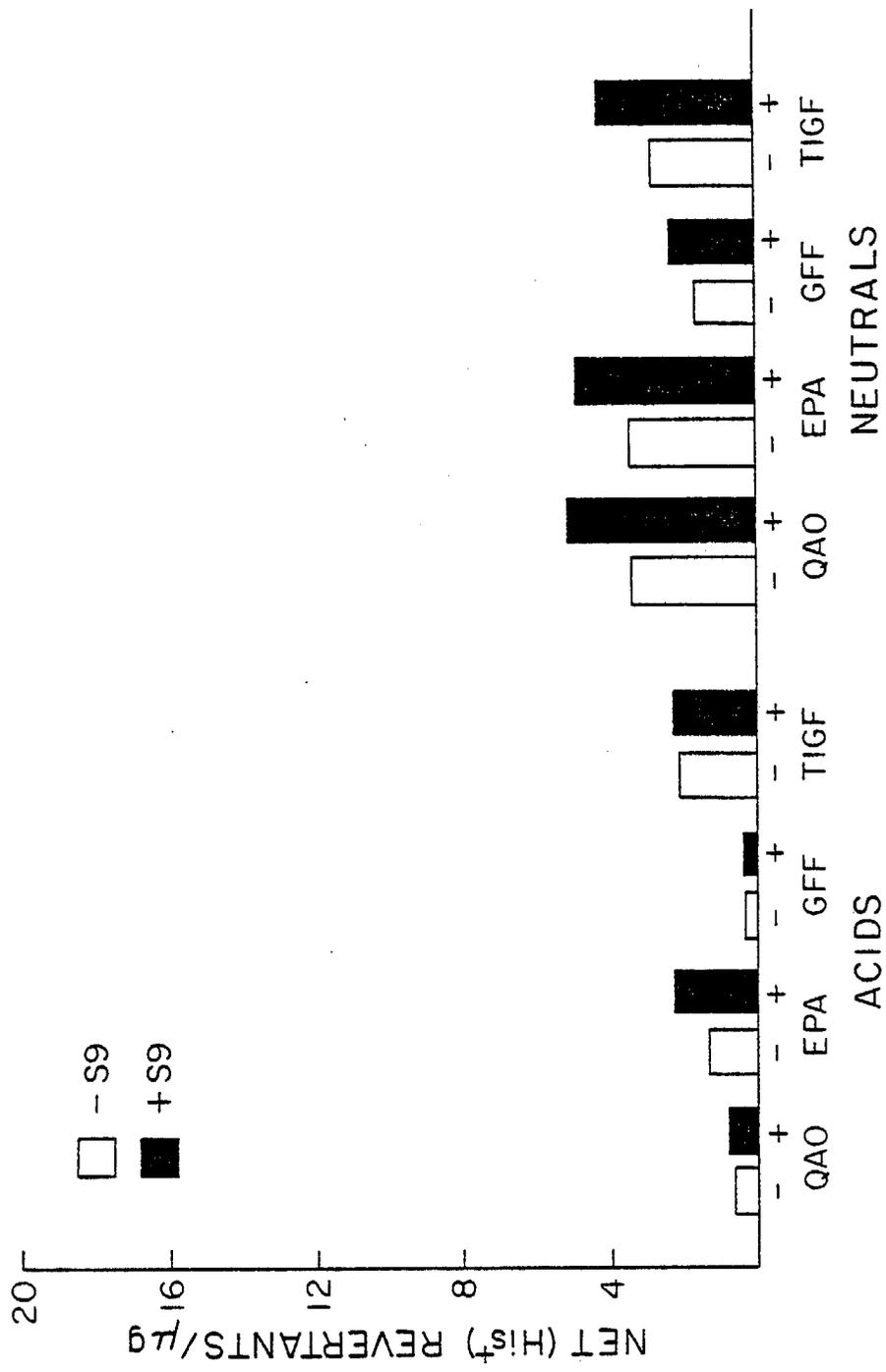


Figure 12. Specific mutagenic activity (revertants/ $\mu\text{g}$ ) on strain TA98 of filter comparison study samples. Set 2, Claremont, CA, June 1-4, 1979.

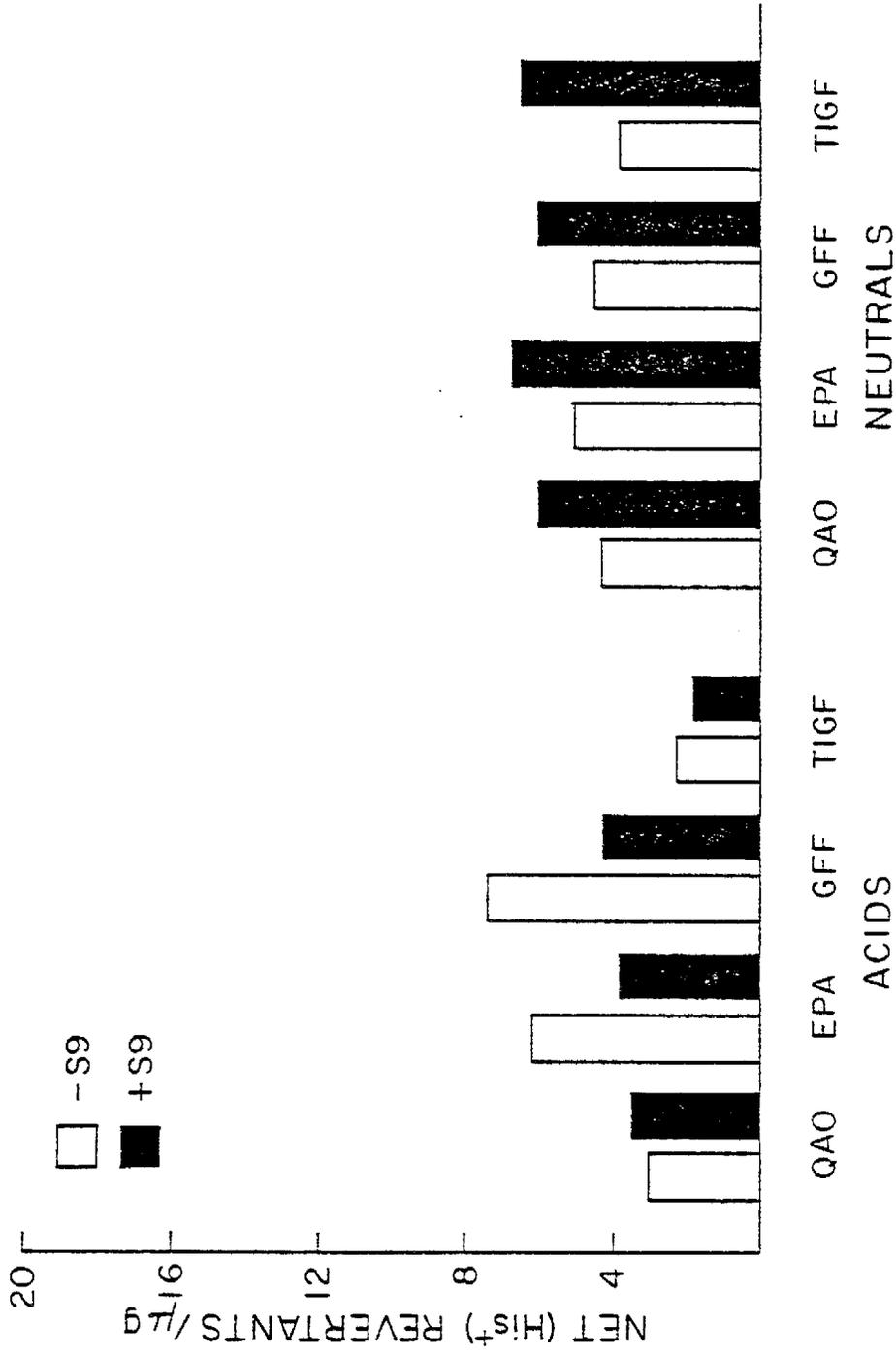


Figure 13. Specific mutagenic activity (revertants/ $\mu\text{g}$ ) on strain TA98 of filter comparison study samples. Set. 3, collected in Claremont, CA, May 23-25, 1979.

This task was carried out on a Waters Associates Model ALC202 liquid chromatography system equipped for gradient elution and dual-wavelength UV absorbance detection. The solvents used were low-residue "distilled in glass" grade, and were purged with and maintained under a helium atmosphere in order to maintain reproducible separation efficiency and detector response. A 4.6 x 250 mm "Ultrasphere I.P." octadecylsilyl bonded reversed-phase column with a theoretical plate count of 15,000 was used in combination with a short guard column, both obtained from Altex Scientific. The separations were carried out at a flow rate of 1.0 ml/min, using a linear solvent composition gradient from 55 to 100% methanol (in water) over a period of 20 minutes which was initiated concurrently with sample injection. The absorbance of the effluent was monitored at 254 and 365 nm.

A third aliquot of each neutral fraction was quantitatively assayed for eight polycyclic aromatic hydrocarbons by combined gas chromatography-mass spectrometry, using a Finnigan Corp. Model 3200 GC-MS fitted with a 40 m x 0.25 mm wall coated (SE-52) open tubular glass capillary column and interfaced with a Finnigan Corp. Model 6100 data system. Standard solutions containing known concentrations of fluoranthene, pyrene, benz(a)-anthracene, chrysene, benzo(e)pyrene, benzo(a)pyrene, perylene, and benzo(ghi)perylene were used to establish response factors relative to 1,3,5-triphenylbenzene (the internal standard) and to verify system linearity and stability during the analysis.

One-sixth of each ether-soluble neutral fraction was dissolved in a dichloromethane solution of the internal standard and a portion of each was injected into the column at 40°C (Grob-type direct injection). Duplicate analyses were carried out on each sample. The column oven was programmed after injection as follows: two minutes isothermal at 40°C, followed by a rise to 140°C at 20°/min and a further increase to 265°C at 4°/min. The linear flow rate of carrier gas (helium) through the column was 30 cm/sec at 30°C.

Ion intensities (70 e.v.) at m/e 178.1, 202.1, 226.1, 228.1, 252.1, 276.1, 300.1, and 306.1 were monitored by the data system; peak areas corresponding to the parent ions of the PAH under investigation were measured, ratioed to that of the internal standard, and converted to sample concentrations by use of the appropriate correction factors.

These measured PAH concentrations were then converted to the ambient concentrations of Table 4 with the use of dilution factors and sampling parameters.

### Results and Discussion

Table 3 presents the sampling dates and durations of each collection set together with the gravimetric data obtained from each set. The raw data include the weights of each ether-soluble fraction, the total weight of the ether-soluble fractions, and the ratio of total ether-soluble extract weight to that of the ambient particulate matter collected on the four filter types in each set. In addition, the fraction masses are given as percentages of the ether-soluble total.

These percentages do not display large variations as a consistent function of filter type within each of the three sets. The gravimetric data, then, do not provide evidence for a collection artifact induced by filter surface composition. However, the ratio of ether-soluble organic extract weight to particulate weight was consistently highest for those samples collected on Teflon-impregnated filters. This effect is not due to interference from residual extractable material remaining on the cleaned filters, since the unexposed TIGF filters used as blanks in this study gave the lowest extractable mass of all of the filter types when treated in the same manner. While the statistical significance of this observed trend is very low, it may indicate that extraction of POM from ambient particulate-loaded TIGF filters is marginally more efficient than from the other filter types studied.

Specific mutagenic activities of the acid and neutral fractions from the three sets are presented in Figures 11-13. These values were obtained from the slopes of the dose-response curves, which all showed good linearity. Data from the basic fractions are not included, since the amounts of material in these fractions were too small to allow accurate quantitation. As has been mentioned previously, the acid-base separation scheme applied to sets 2 and 3 differed from that used for set 1 in that 10% potassium bicarbonate solution was substituted for 1N sodium hydroxide in the isolation of acidic substances from the crude extract. This change was made in order to diminish the degree of exposure of the samples to strongly basic media. These are atypical of the atmospheric environment

Table 4. Calculated ambient polycyclic aromatic hydrocarbon concentrations (ng/m<sup>3</sup>) during the filter comparison study, Claremont, CA, Spring 1979.

	Sampling Time, Hours	FA	PY	BaA	CH	BeP	BaP	PER	BghiP
<u>Set #1</u>									
QAO	48	1.12	1.12	1.23	1.47	1.77	1.16	7.54	3.68
EPA	73	0.65	0.57	0.58	0.70	0.83	0.44	0.06	2.08
GFF	73	0.65	0.67	0.46	0.63	0.71	0.36	1.33	1.51
TIGF	73	0.80	0.79	0.44	0.65	0.69	0.28	0.08	1.45
<u>Set #2</u>									
QAO	74	0.45	0.45	0.26	0.62	0.59	0.22	-	1.31
EPA	74	0.55	0.45	0.44	0.57	0.62	0.17	0.59	1.31
GFF	74	0.50	0.48	0.37	0.61	0.67	0.25	-	1.55
TIGF	74	0.73	0.69	0.51	0.73	0.73	0.26	0.04	1.67
<u>Set #3</u>									
QAO	45	0.82	0.86	0.65	0.98	1.04	0.47	0.06	2.55
EPA	45	0.61	0.67	0.59	0.98	1.24	0.67	0.29	2.94
GFF	45	0.88	0.96	0.61	1.16	1.18	0.35	-	2.94
TIGF	45	0.75	0.73	0.61	1.02	1.20	0.43	0.05	3.14

FA = Fluoranthene, PY = Pyrene, BaA = Benz(a)anthracene, CH = Chrysene; BeP = Bezno(e)pyrene, BaP = Benz(a)pyrene, PER = Perylene, BghiP = Benzo(ghi)pyrene.

from which the samples were collected and they might be expected to alter the overall composition of the extracts. The composition of the "acidic" fractions was expected to respond to this change in the base. When NaOH was used, the "acidic" fractions were expected to contain those components of the mixture with pKa's of less than 12 (carboxylic acids, sulfonic acids, phenols, enolizable ketones and aldehydes, etc.) with water-soluble sodium salts; the bicarbonate procedure was expected to result in an "acidic" fraction containing species with pKa's of less than 7 (carboxylic and sulfonic acids and other relatively strong acids), again with water-soluble salts. Potassium was employed as the counter-ion in order to enhance the solubility of these salts. Most phenols and other weakly acidic species present in the extracts were expected to remain in the "neutral" fraction in the latter case.

Inspection of the mutagenicity data for set one (NaOH) (Figure 11) reveals no substantial difference in activities of the samples derived from the various filter types.

The mutagenic activities of the fractions derived from sets two and three showed significant differences, particularly in the acidic fraction response. The mutagenic activities of the neutral fractions were comparable, both with and without metabolic activation, within both sets with the exception of the GFF sample of set two. Since this phenomenon was not observed in the other two sets, and the reported activity of this GFF sample was approximately half that of the other samples in this set, it seems likely that this result was due to a error during weighing, sample resuspension in DMSO or dilution for the Ames testing. Although we have been unable to trace any such error, this conclusion is supported by the results of the HPLC and GC-MS analyses (vide infra). Variation in the measured activities of the acidic fractions of sets two and three was quite pronounced, but the data do not support any conclusions regarding the effect of filter type on these values. This "scatter" in the mutagenicity of the acidic fractions obtained using  $\text{KHCO}_3$  as the extracting base appears instead to reflect a lack of reproducibility in that procedure (Section V).

HPLC analysis of the neutral fractions of this study produced approximately 50 resolved chromatographic peaks which were recorded at two observing wavelengths (254 and 365 nm) over a total analysis time of 36

minutes. Visual comparison of the chromatograms revealed a high degree of qualitative uniformity among the samples within each of the three sets. The most notable exception to this uniformity was a small peak which appeared at 20.3 minutes in the QA0 sample from set two. Variations in relative peak heights were quite pronounced among the three sets, reflecting the presumed variability in the composition of the ambient aerosol during the three sampling periods. Relative peak height variability within each of the three sets, however, was smaller in magnitude and fell within the limits imposed by the small variations in chromatographic conditions encountered when solvent composition programming is employed. This "fingerprinting" method, then, failed to demonstrate any differences among the samples attributable to an effect of filter composition.

Table 4 presents the levels of selected polycyclic hydrocarbons in the sampled ambient air as determined by combined gas chromatography-mass spectrometry analysis of the neutral fractions. The precision of this analysis is estimated to be  $\pm 30\%$ . Inspection of the data reveals no consistent or significant trend among the filter types examined. The large scatter in observed perylene concentrations does not correlate with filter type and must result from an unknown source of variability in our experimental procedure. An effect of sampling time may be reflected in the higher values observed in the sample collected on the quartz filter of set one. This would be consistent with evaporative or oxidative losses from the filters during sampling, resulting in lowered values in those filters exposed for longer periods. Alternatively, the ambient air during the first 48 hours may have been more heavily loaded with particulate PAH than during the remainder of this sampling period.

In summary, these methods have failed to distinguish a "filter artifact" ascribable to an influence of filter composition. It is possible that a "leveling effect" resulted from the long collection times used in this study and masked such an artifact. We are continuing studies directed toward this problem under separate funding. In the absence of other selection criteria, the Teflon-impregnated glass fiber filters were chosen for use in the measurement of the geographical distribution of airborne particulate mutagenic activity based on the gravimetric results, which indicated (a) higher extraction efficiency for this filter over other types examined, and (b) the expected lack of reactivity of the Teflon surface.



## V. EXAMINATION OF THE REPRODUCIBILITY OF THE SAPRC EXTRACTION AND FRACTIONATION PROCEDURES

Interpretation of the data generated in this geographical distribution study requires some estimate of the errors involved in the sampling, extraction, extract processing, and Ames test procedures used. With the recognition that a part of the value of this work may be determined by the repeatability of the methods used here, we have performed several experiments directed toward quantitative measurements of some of these errors.

Extraction/Fractionation Procedure Reproducibility. This experiment was designed to examine the reproducibility of our procedure for recovery of ether-soluble organics from particulate-loaded filters. Eight samples were collected simultaneously on washed and fired Gelman A-E glass fiber filters. These were quartered and regrouped into four samples in a way intended to average out any sampling error. Therefore, each sample was the equivalent of two filters. These samples, and a blank composed of two washed and fired glass fiber filters, were subjected to our extraction/fractionation procedure (using  $\text{KHCO}_3$ ). The results are presented in Table 5. (There are three samples listed in the table because the fourth sample was lost during the procedure.)

Examination of the weights of the fractions expressed as percent ether-soluble organics shows the reproducibility of the present acid/base/neutral fractionation procedure to be quite good. There is larger variability in the ether-soluble organic total fractions, however. Since the filter scrambling should have averaged any sampling error, this variability is most likely due to variations in "super" solvent (1:1:1 benzene, dichloromethane and methanol) extraction efficiency or small errors in accurately dividing the filters into quarters.

These four sets of acid, base, and neutral fractions were also assayed for direct and activatable mutagenicity toward TA98, using the methods described in Section III. The results of this assay are presented in Figure 14. The specific activities obtained from these samples paralleled those of the acid/base/neutral fractionation scheme used in the filter comparison study (Section IV) when  $\text{KHCO}_3$  was used, in that the acid fractions replicated poorly in the Ames test. A check of the Ames test procedure using a mutagenic carboxylic acid, 4-nitro-2-furoic acid, gave reproducible

Table 5. Extraction/fractionation procedure reproducibility. Recovery of ether-soluble organics from particulate-loaded filters.

Sample	Acids		Bases		Neutrals		Total Sum mg	ESOx100 TSP
	mg	(% Total)	mg	(% Total)	mg	(% Total)		
#1	75.828	(61.6)	1.177	(0.96)	46.025	(37.4)	123.030	9.7
#2	75.583	(61.1)	1.257	(1.06)	44.989	(37.9)	118.829	9.4
#3	69.936	(60.5)	1.439	(1.24)	44.246	(38.3)	115.621	9.1
Blank	2.728	(68.0)	0.127	(3.16)	1.159	(28.9)	4.014	-

ESO - ether soluble organics

TSP - total particulate weight (collected) which was 1.2656 g

Sample Origin

Hi-vol, GFF filters

Location: Claremont, California

Flow rate: 40 CF/m

Time: 70 hr, 6/22 to 6/25/79

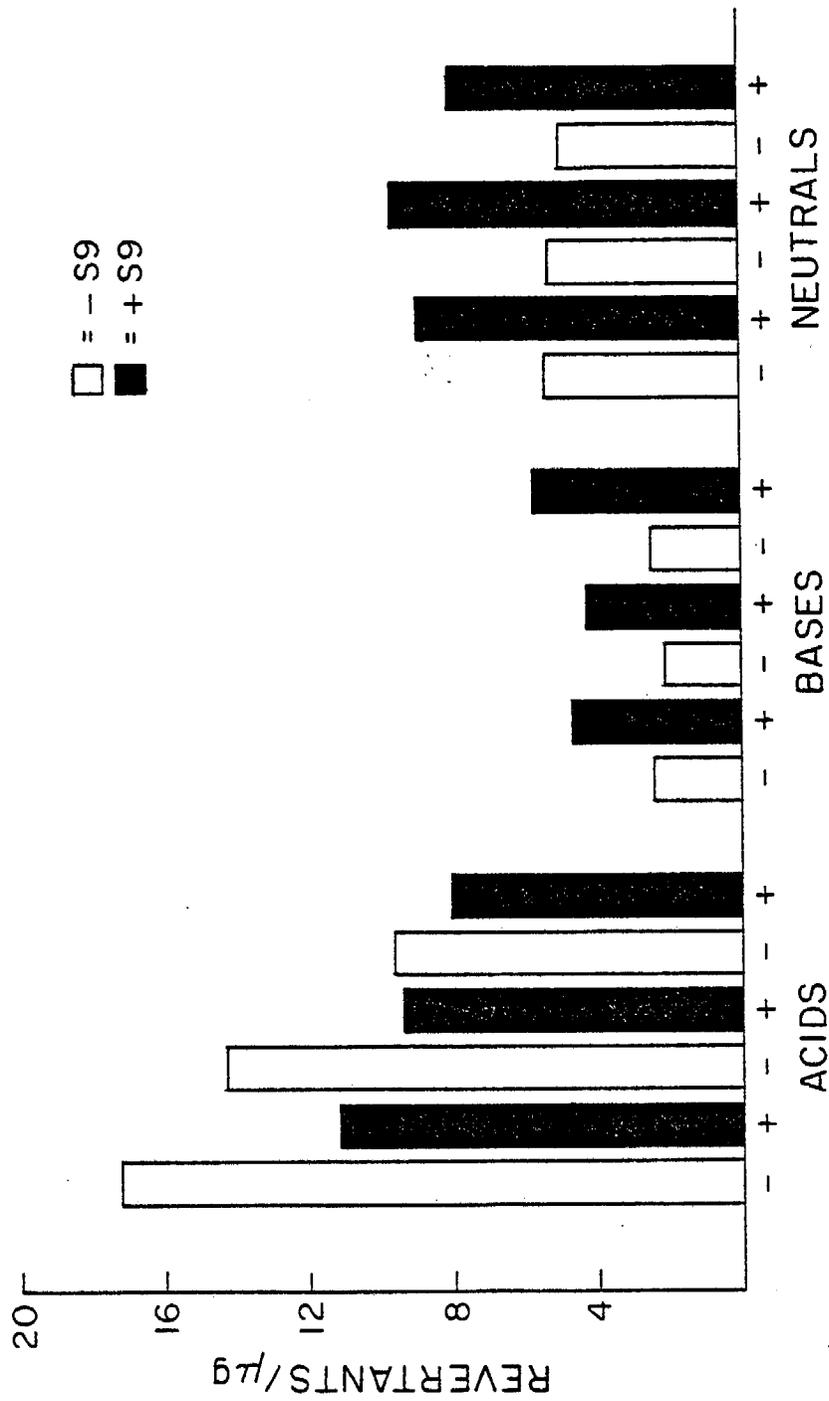


Figure 14. Extraction/fractionation procedure reproducibility. Comparison of specific mutagenicity (revertants/μg) on strain TA98 of samples collected in Claremont, CA, June 22-25, 1979.

and linear dose responses when tested with TA98; thus, any variability associated with the Ames assay of acidic mutagens could be discounted. The level of reproducibility attainable when the weak base  $\text{HCO}_3^-$  was used to obtain an acidic fraction of ambient particulate extracts was judged to be insufficient to allow meaningful comparisons to be made among the geographical distribution samples. While the reproducibility was improved when a stronger base was used to obtain an "acid" fraction, we believe that such harsh treatment is incompatible with our desire to minimize changes in the chemical composition of ambient particulate extracts during sample processing. In principle, the same objections can be raised regarding any fractionation scheme which subjects an extract to conditions substantially different from the mildly acidic, oxidizing environment of the ambient air from which these samples are derived. For these reasons we turned to an examination of the accuracy and reproducibility attainable when "total extracts" are used in an assay of this type.

Extraction and Sample Handling. The solvent system used in this work has been shown to be >95% efficient in extraction of organic carbon from ambient particulate by combustion analysis (Grosjean, 1975). A preliminary investigation, in which the mass and mutagenic activity of successive extracts of a filter heavily loaded with atmospheric particulate were compared, gave essentially the same result. One washed and fired (450°C) glass fiber filter used to collect ambient particulate for 70 hours at Harvey Mudd College in Claremont, California (0.6673 g TSP) was extracted twice sequentially by our present method. The two extracts, EE1 and EE2, as well as a solvent blank, were reduced in volume in vacuo, transferred to tared vials, evaporated to constant weight with a stream of dry nitrogen, and weighed. A portion of each sample was examined by reverse-phase HPLC, and the remainder was submitted for Ames testing. Extracted mass and specific activity data are presented in Table 6.

The second extraction of this heavily loaded glass-fiber filter, intended to represent a "worst case" situation, produced an additional 9% of material, with a lower specific mutagenicity, both direct and acti-vatable, than the first extract. The chromatograms produced by the two extracts were qualitatively similar, with the second extract showing

Table 6. Extraction/fractionation procedure reproducibility. Efficiency of extraction of mass and mutagenic activity from particulate-loaded filters.

Extract	Mass (mg)	Specific Activity (rev/ $\mu$ g)	
		-S9	+S9(2%)
EE1	233.23	0.71	0.421
EE2	21.13	0.38	0.35
Solvent blank	0.52	inactive	

a relative enrichment in the concentration of low polarity components of the mixture. This is consistent with the Ames test results, in that the activatable specific mutagenicity (presumably due to low polarity compounds such as the parent and alkylated PAH) decreased less than the direct activity, which has been associated with chromatographic fractions of higher polarity in the second extract. Inclusion of the data from the second extract in a calculation of airborne mutagen density would, in this single case, increase the measured levels of direct and activatable mutagenicity by 5% and 7%, respectively. The results of this study are obtained from a single extraction of each sample filter. We are continuing to investigate the feasibility and value of more thorough extraction by means of additional studies of the type outlined above.

#### Reproducibility of "Total Extract" Mutagen Assay

Procedures Using Teflon-Impregnated Glass Fiber Filters. An experiment was designed to evaluate the reproducibility of mutagenic assay of ambient particulate, using our procedures for obtaining, processing and Ames testing crude "super solvent" extracts of four identical ambient particulate samples collected on Teflon-impregnated glass fiber filters. Collection of the samples was carried out at El Monte from 10:25, 10/2/79 to 13:43, 10/3/79 using the SAPRC "megasampler" and four pre-extracted TIGF filters. The "megasampler," constructed under EPA funding, consists of four separate samplers mated together, and samples at the same face velocity

as a standard hi-vol. Due to its larger size, however, it is capable of processing four times the flow (160 c.f.m. per filter) of a standard hi-vol. The loaded filters were not weighed due to their large size (16" x 20"), but were instead immediately extracted by our usual procedure. Ten percent of the extract was evaporated to constant weight ( $\pm 1\%$ ) and submitted to the microbiology group. This sample size corresponds to that obtained from an 11-hour collection using a standard hi-vol, so the results can be compared to the 12-hour collections used for the geographical distribution study (Section VII).

TA98 was plated in triplicate with 0, 4 and 10% S9 (v/v) at extract doses of 1, 10, 20, 40, 60, 80, 100, 150, 250 and 500  $\mu\text{g}/\text{plate}$ .

Use of the 10% S9 level led to suppression of mutagenicity. Slopes of the dose response curves were calculated from the values obtained with doses of 0-100 or 0-150  $\mu\text{g}/\text{plate}$  (good linearity was observed over this concentration range, see Figure 15). The results of this analysis are shown in Table 7, together with TA98 responses to a solvent residue sample and two standard mutagens, 2-nitrofluorene and 2-aminoanthracene.

Agreement among the four samples was very good, approaching the reproducibility of our Ames test procedures when applied to pure standard mutagens. On the basis of these results, we have (conservatively) estimated the standard error of our procedures as applied to total extracts of ambient particulate at  $\pm 15\%$ .

This figure strictly applies, of course, only to equivalent samples collected concurrently; variations in the composition and physical properties of the samples collected in the distribution study could adversely affect the precision of the analysis. The effects of such variations in sample composition are largely indeterminable but we believe that such effects are small in comparison to the other sources of error in this analysis.

Several other aspects of the procedures used in the study described in Section VII deserve comment, and these issues are discussed below.

Sampling. The filter surface (Teflon) chosen for this work is generally thought of as chemically inert, so that we expect minimum interference with the analytical process from surface-catalyzed alteration in POM composition. The extent of any artifacts induced by surface

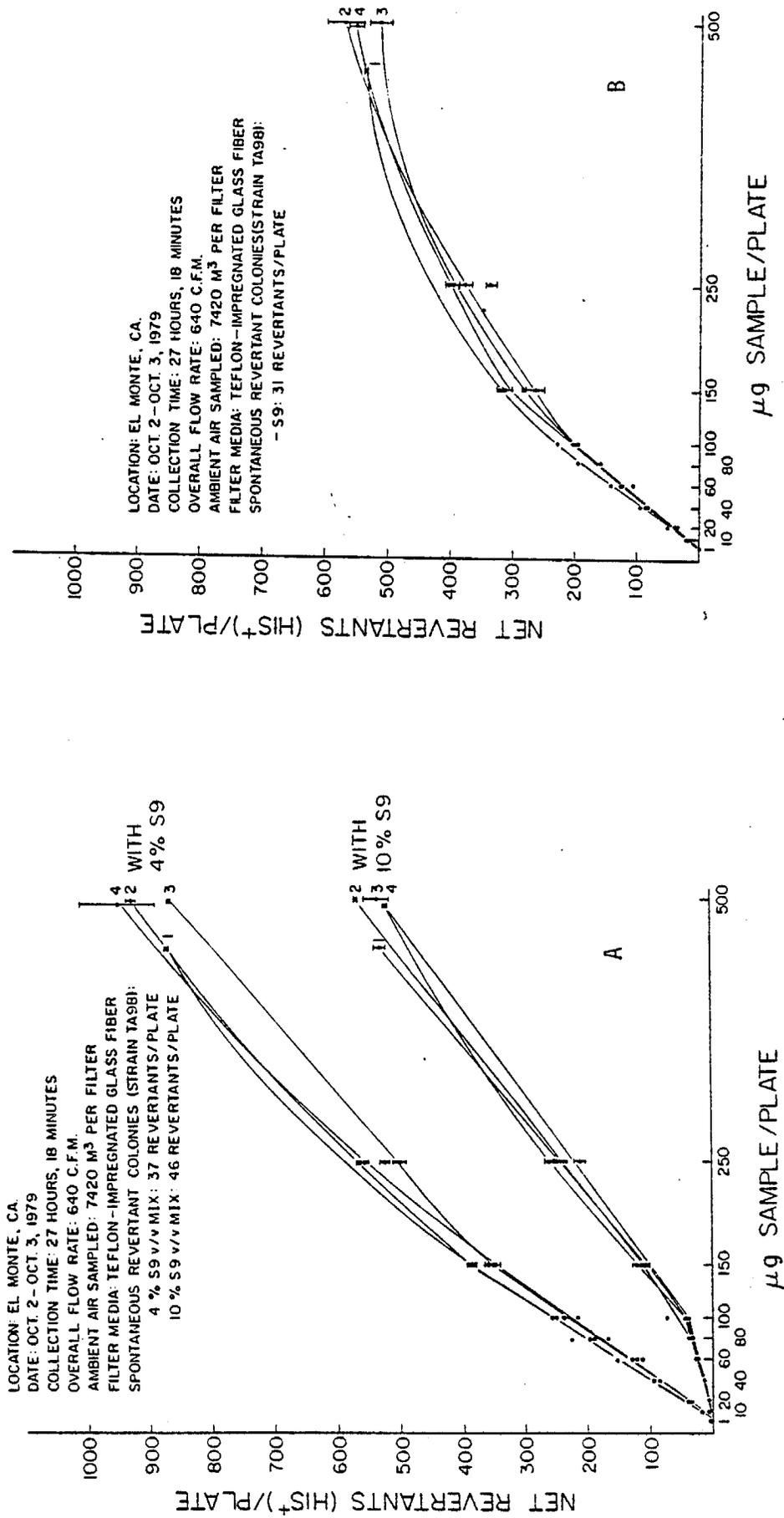


Figure 15. Mutagenic activity on strain TA98 of four samples of ambient airborne particulate collected concurrently on the SAPRC megasampler. (A) Mutagenic activity without metabolic activation. (B) Mutagenic activity with metabolic activation.

Table 7. Reproducibility of the results of the mutagenic assay on TA98 of the total extracts of four samples collected concurrently using the SAPRC megasampler, El Monte, CA, October 1979.

Sample Number	Extract Mass (mg)	Specific Activity (Ref/ $\mu$ g)		Calc. Mutagen Density (Rev/ $m^3$ )	
		-S9	4% S9	-S9	+S9
1	22.471	2.4	2.6	73	79
2	23.257	2.0	2.6	63	81
3	24.460	2.0	2.4	66	79
4	23.249	2.1	2.5	66	78
solvent blank	0.556	-	-	-	-
2NF		450	-	-	-
2-AA		-	~2800	-	-
Ave. (1-4)	23.359	2.1(25)	2.5(25)	67	79(.25)
N-1 (%)	3.5	8.9	3.4	6.3	1.6

Ambient air sampled per filter: 7420  $m^3$

Total collection time: 27 hrs, 18 min

Filter type: Teflon-impregnated glass fiber

adsorption and subsequent reaction of gas-phase pollutants such as nitric acid, nitrogen and sulfur oxides, and ozone with the collected POM, or by evaporative losses of mutagenic material during sampling, remain unknown, and our results reflect the effects, if any, of such phenomena. The inclusion in the sample of particles outside the respirable size range should not interfere with assessment of the mutagenic hazard associated with the sampled air, since the great majority of the activity is known to be associated with particles which are respirable. The inclusion of the larger particles in the sample does have an effect on the values we obtain for mutagenic activity on a particulate mass loading basis, however, since particle size distribution can vary with location and sampling time.

The sampling period used, 12 hours, represents a compromise of the ideal of instantaneous and continuous monitoring (which is possible for many pollutants) due to the sample size requirements of the Ames assay and our desire to minimize difference in history among the samples during processing. We believe, however, that 12-hour samples are superior to longer collections in terms of fidelity to real world conditions, and are investigating the possible benefits (apart from improved time resolution) to be gained from shorter sampling periods under EPA funding.

Solvent Reactivity. The solvent mixture employed in this work incorporates a protic, nucleophilic alcohol (methanol), a geminal dihalide (dichloromethane), and an alkylated aromatic system (toluene). These chemicals have the potential of reacting with extracted organic material, with inorganic constituents of the sample, or with each other, to produce products which would represent artifacts of the extraction procedure. For example, methanol can react with functional groups which are susceptible to nucleophilic displacement or addition, participate in solvolysis reactions, and react with free acids to form methyl esters. Dichloromethane is an alkylating agent, although a poor one, and could react with strongly nucleophilic constituents of the sample (amines, for example); toluene can undergo electrophilic substitution in the presence of Lewis acids, again producing undesired reaction products. The procedure used here is designed to minimize any such effects on the composition of the extract through the avoidance of elevated temperatures and minimization of sample-solvent contact time. No component of the extracted material which can be attributed to such reactions has yet been identified in this laboratory. When the extract is fractionated into acidic, basic, and neutral fractions, for example, carboxylic acids can be identified by GC-MS (after derivatization) in the acidic fraction, while the corresponding methyl esters are not detected in the neutral fraction.

Solvent Removal. Complete removal of the solvent used for extraction of the collected particulate is necessary in order to avoid toxic or co-mutagenic effects arising from the presence of residual solvent and in order to accurately weigh the extracts.

There is a real possibility of evaporative losses from the extract during this process. We have previously shown that rotary evaporation

under reduced pressure results in no loss of mutagenic material when compared to Kuderna-Danish concentration. The final drying stage, during which the last traces of solvent are removed by a stream of dry nitrogen, is currently being evaluated for loss of mutagenic material by passage of the exhaust gas through a solid adsorbent (Tenax) bed and Ames assay of extracts of the adsorbent.

## VI. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY SEPARATION AND CHEMICAL ANALYSIS OF AMBIENT PARTICULATE EXTRACTS

Research at SAPRC directed toward the isolation and identification of directly mutagenic species present in the Los Angeles ambient particulate has progressed during the past year, benefiting from the application of high performance liquid chromatography (HPLC) to the problem.

Experimental. A Waters liquid chromatograph equipped for gradient elution has been used in the reversed-phase mode (C-18  $\mu$ -bondapack columns) for the resolution of neutral fractions derived from an acid/base/neutral separation of ambient particulate extracts. The resolved fractions exiting the HPLC have been tested for mutagenicity, and the most active fraction has been purified further and investigated by mass spectroscopy and other physical methods.

Results and Discussion. Preliminary evaluation of the HPLC/Ames test approach was performed with a neutral sample (31.14 mg) obtained from a daytime collection in downtown Los Angeles on February 22-27, 1978 using washed and fired glass fiber filters. The sample was separated using a methanol/water gradient elution on a semi-preparative C-18 column into ten fractions collected at four-minute intervals. These fractions were then submitted for testing with the Ames mutagenicity assay, and levels of direct and activatable mutagenicity were determined for each. The results are shown in Figure 16, as total revertants per fraction, calculated from the fraction mass. [These graphs must be viewed as approximate, reflecting our experience with variability in the Ames test (see Section IV) as well as gravimetric error.]

A second more complete HPLC separation was carried out on 40.3 mg of the neutral fractions from the nighttime collections during the same period (February 22 through 27, 1978). Part of the aromatic hydrocarbon content of the sample was removed by partition of the neutral fraction between methanol and cyclohexane. The more polar constituents of the sample (methanol layer) were then resolved into 24 separate fractions, giving the mutagenicity profile displayed in Figure 17. Also, absorption, fluorescence, and chemical ionization (methane) mass spectra were obtained for each fraction in order to provide information on the nature of the compounds present. The mass spectra suggested the presence

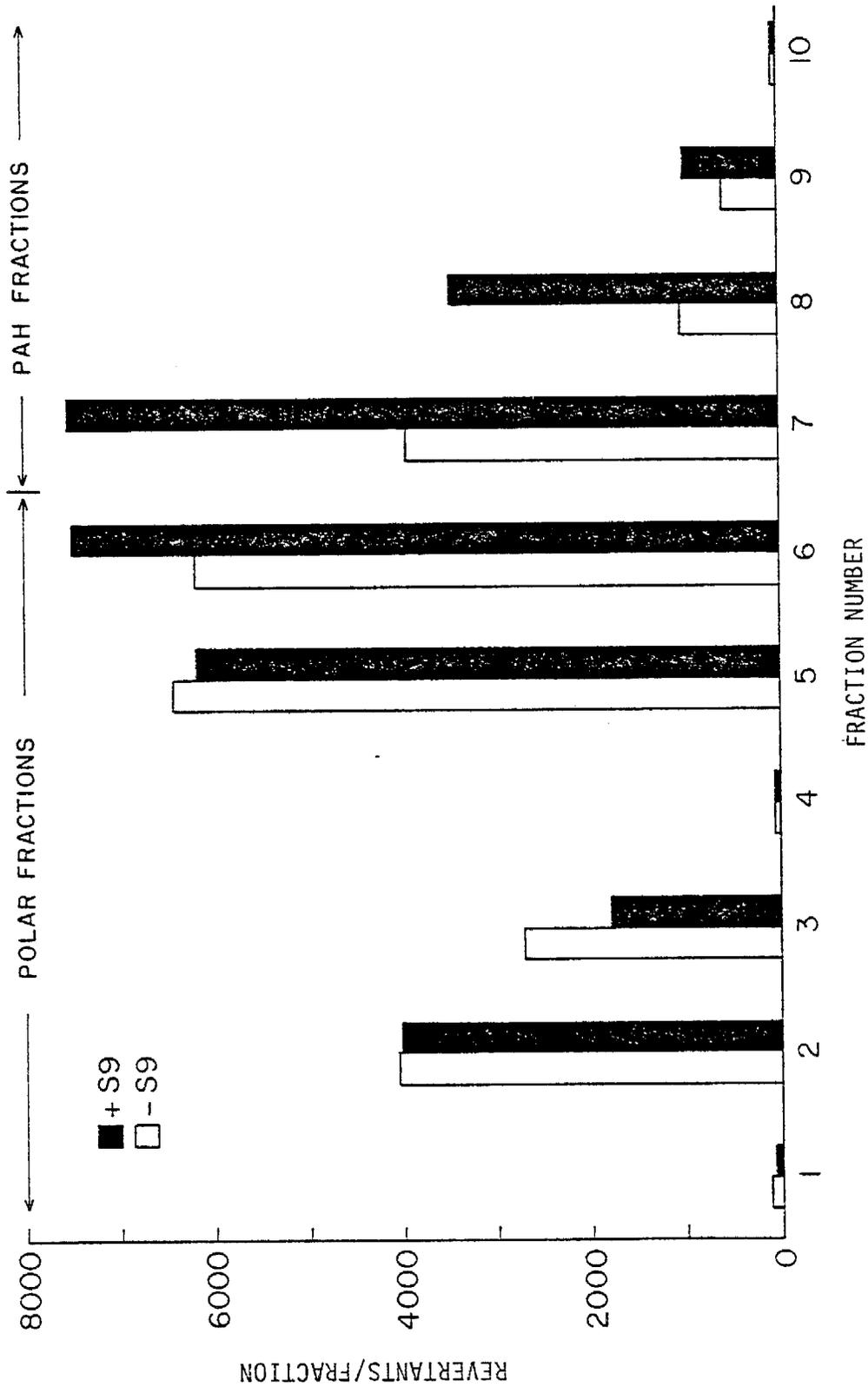


Figure 16. Total mutagenicity (revertants/fraction) with strain TA98 of HPLC fractions of day neutrals of ambient particulate collected on glass fiber filters in downtown Los Angeles, February 22-27, 1978.

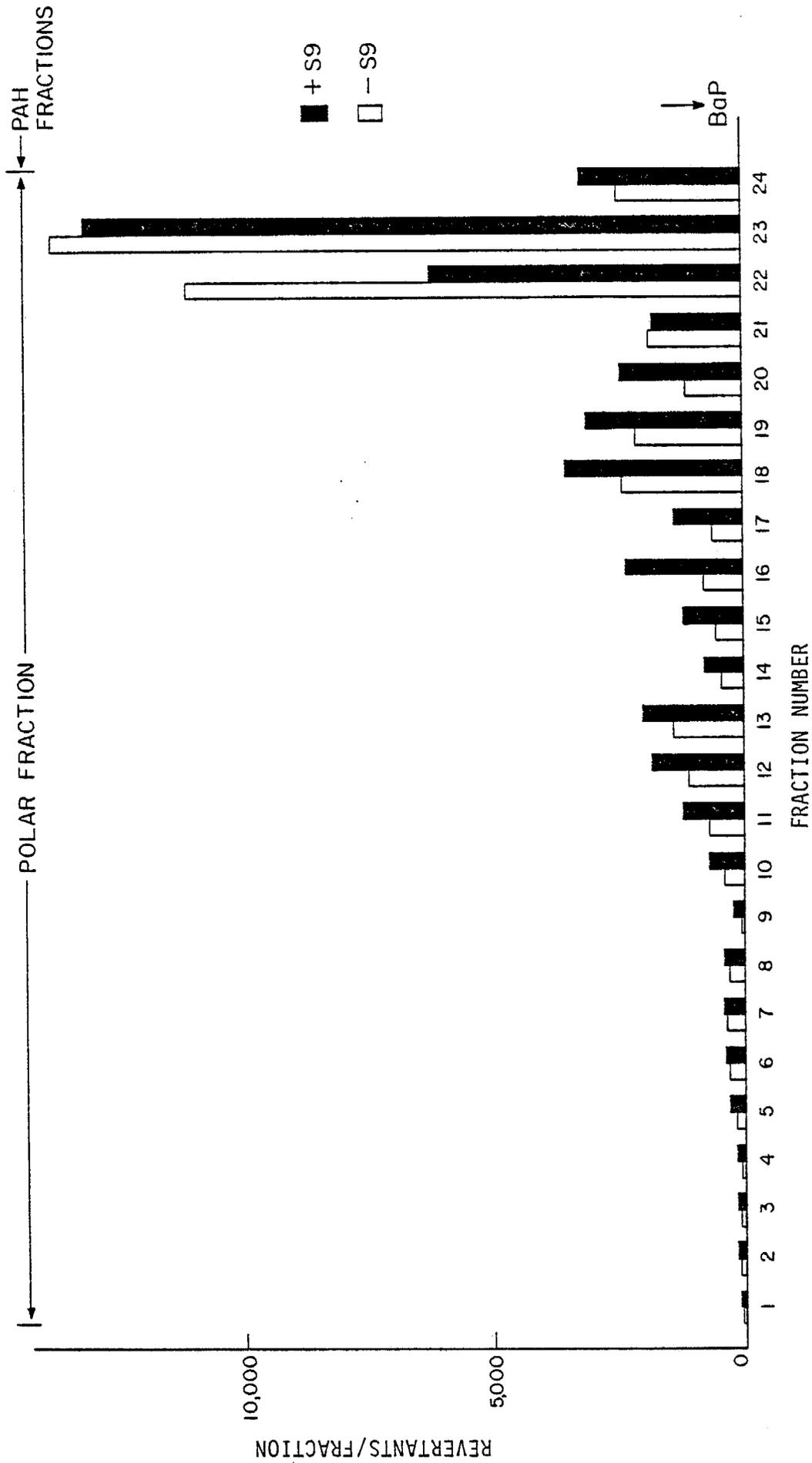


Figure 17. Total mutagenicity (revertants/fraction) with strain TA98 of HPLC fractions of night polar neutrals of ambient particulate collected on glass fiber filters in downtown Los Angeles, February 22-27, 1978.

of several types of PAH oxidation products (Table 8). Molecular ions were observed corresponding to those of several parent hydrocarbons (m/e 228, 252, 278 and 300), quinones (m/e 208, 232, 258 and 282), ring-opened products such as dialdehydes, ketoaldehydes or anhydrides (m/e 210, 234, 272, 284 and 310), dihydrodiols (m/e 236, 250, 286, 310 and 312) and phenols or epoxides (m/e 218, 232, 244, 268 and 316). These were found to be major ions in the most active fractions, and the masses corresponding to derivatives characteristic of benzo(a)pyrene and its isomers (C<sub>20</sub>H<sub>12</sub>) were especially prominent.

The procedure used for the February, 1978, nighttime neutrals was then applied to the neutral fraction (62.1 mg) derived from particulate samples collected on quartz fiber filters in Claremont, CA, during the period from August 10 to September 18, 1978. Thirty-two fractions were collected from the HPLC separation and assayed for mutagenic activity, with results as shown in Figure 18. The most active HPLC fractions in all three experiments had the same retention time relative to BaP (0.83 - 0.87). Three fractions corresponding to this range of relative retention

Table 8. Molecular weights of several polycyclic aromatic hydrocarbon derivatives.

PAH	Parent MW	Epoxide or phenol	Dione	Dial or Keto-al	Dihydro-diol
A, Ph	178	194	208	210	212
Pyrene	202	218	232	234	236
BF	216	232	246	248	250
BaA, CH, T	228	244	258	260	262
BaP, BeP,	252	268	282	284	286
BkF, PER					
BghiPe	276	292	306	308	310
DBA	278	294	308	310	312

A = anthracene, BaA = benz(a)anthracene, BF = benzofluorene, CH = chrysene, BkF = benzo(k)fluoranthene, BghiPe = benzo(ghi)perylene, PER = perylene, T = triphenylene, DBA = dibenzo(ah)anthracene.

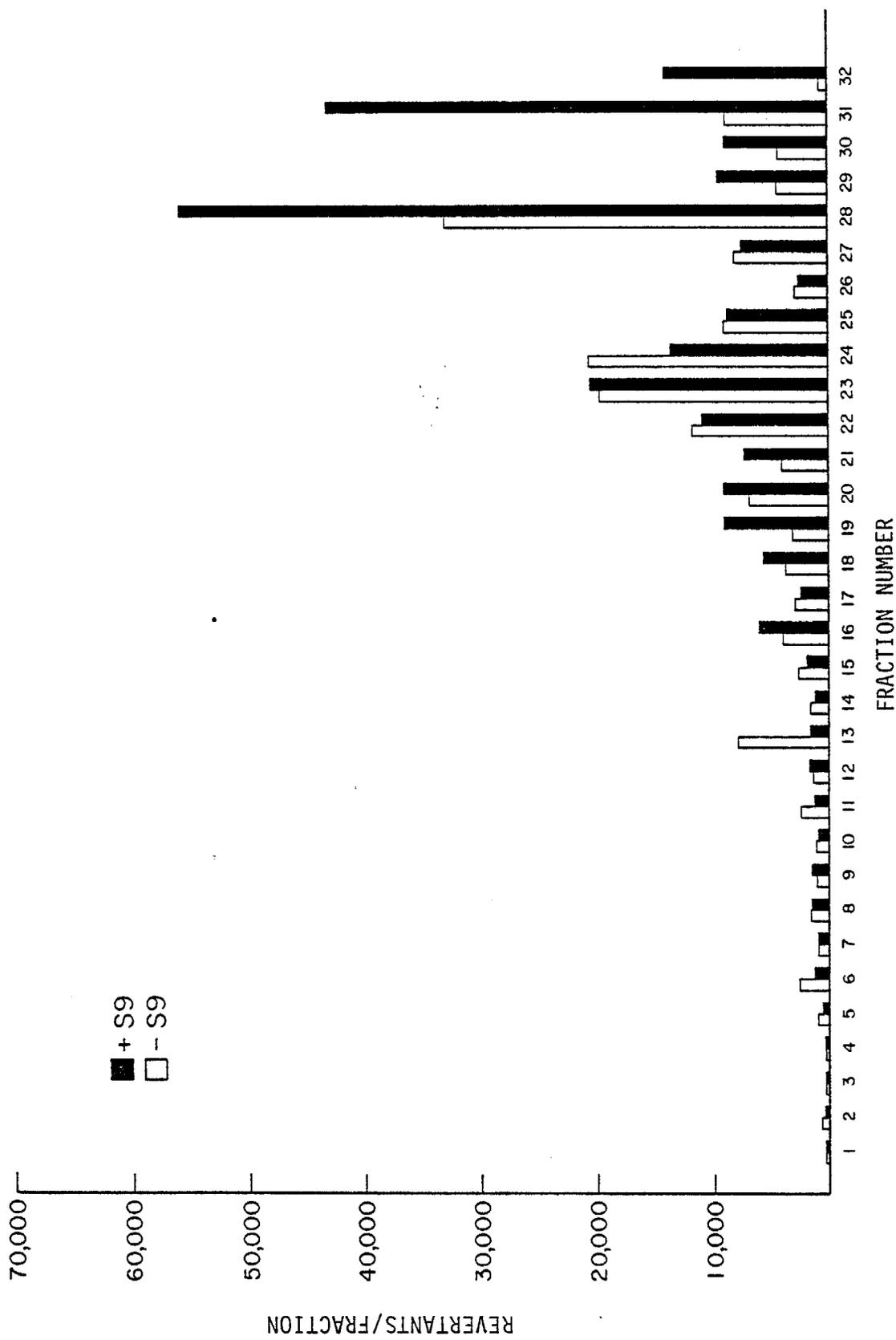


Figure 18. Total mutagenicity (revertants/fraction) with strain TA98 of HPLC fractions of the neutrals of ambient particulate collected on glass fiber filters, Claremont, CA, September 10-18, 1978.

times were then subjected to further separation on two analytical (30 x 0.24 cm) C-18 columns. The fractions obtained by this procedure still contained several components. The major constituent of the most active fraction gave mass spectra indicating a molecular weight of 254 [EI: m/e 254 ( $M^+$ ), 226 ( $M^+-CO$ )]; [CI ( $CH_4$ ): m/e 283 ( $M^++C_2H_5$ ), 255 ( $M^++1$ )]. A compound or compounds with molecular weight 270 was also present. The same relative retention times and mass spectral ions were observed when directly mutagenic photooxidation and ozonation product fractions of BaP were examined, suggesting that some of the direct activity of ambient particulate extracts is due to the presence of BaP oxidation products. Recently, further purification of the fraction with compounds of molecular weight 270 together with high resolution mass spectroscopy has revealed the presence of two contributors to this fraction of molecular formulae  $C_{19}H_{10}O_2$  (major) and  $C_{20}H_{14}O$  (minor). Further studies on the structures of these and other directly mutagenic fractions is continuing.

We have recently purchased two HPLC reversed-phase columns with approximately twice the resolving power of those used in this work, and are investigating the compatibility of an acetonitrile/water gradient with the Ames test. We hope to improve our separation efficiency by these means. Investigation of the compatibility of reagents used in ion-pair chromatography with the Ames test will also be undertaken, in order to enable similar separation and assay of the acidic fraction of POM extracts.

VII. GEOGRAPHICAL AND TEMPORAL DISTRIBUTION OF MUTAGENIC ACTIVITY  
ASSOCIATED WITH AIRBORNE PARTICULATE MATTER

Experimental

1. Sampling Sites and Collection Protocol. During the filter comparison study, it was observed from the three collection sets that there was a significant variability of mutagenic activity on a weight basis for a given filter type among the collection periods even though the days were similar in air quality (based on ozone concentrations). For this reason, the field samplings for this program were redesigned to be carried out concurrently at all sites. This necessitated lowering the number of sites to eight, and reliance on SCAQMD stations for the measurement of pollutant concentrations.

Eight sites were chosen to be representative of well-defined types of air pollution found across the South Coast Air Basin, as shown in Figure 1 (see Section I). In addition, six of the locations used were near SCAQMD monitoring stations so that their air quality data could be utilized. Two high volume air samplers (hi-vol) were installed at each site.

A pair of sites was used to study automotive emissions by concurrently sampling upwind and downwind from a major freeway. The original choice was the West Los Angeles location selected by the EPA and ARB researchers for the Los Angeles Catalyst Study (LACS). Prevailing westerly winds bring in relatively unpolluted air, as there is only light to moderate traffic density between this area and the Pacific Ocean, about 8 kilometers to the west. Unfortunately, the LACS monitoring stations and electrical power connections to them had been removed sometime before the planned summer sampling in 1979. This necessitated slight changes in location. The upwind site (#1) was located at the VA hospital on the roof of a gymnasium (next to the Building and Grounds Department) about 100 meters northwest of the former LACS station. The downwind location (site #2) was in the West Los Angeles baseball park, next to the I-405 fence, two blocks west of Westwood Village. Here the hi-vols were at ground level, necessary because of the lack of electrical outlets near buildings (the pitching machine had the only outlet in that area). This location was about 50 meters northwest of the former site of the LACS trailer. Ambient air monitoring at the upwind and downwind sites could not be

done because building space was not available, and the electrical power demand from the samplers was already high. General air quality data was obtained at the SCAQMD West Los Angeles monitoring station, about 2 kilometers to the southeast.

Sampling was done downwind of three types of industrial emissions: petroleum refiners, a power plant, and a steel mill. The Long Beach area includes several major petroleum refineries and coastal westerly winds bring relatively clean marine air past the refineries and to the sampling site (#3) on the roof of Fire Station #9, 3917 Long Beach Blvd. This building is three blocks north of the SCAQMD Long Beach monitoring station.

The Huntington Beach power plant was chosen as an example of these emissions. Again, incoming clean air from the ocean provides a low background and the absence of other major primary emission sources was expected to simplify data interpretation. The hi-vols were located on the roof of Costa Mesa Fire Station #1 at the corner of Addams and Royal Palm Streets (site #4). This site is about 5 kilometers northeast of the power plant, enough distance for the plume to reach ground level. The SCAQMD Costa Mesa monitoring station is 10 kilometers to the southwest.

Since there is only one steel mill in the SCAB, this sampling site (#7) was located downwind from that steel mill in Fontana. The roof of Redwood Elementary School was used as a platform for the samplers. This school, on Redwood Avenue between Arrow Highway and Whittram Street, is about 2 kilometers northeast of the steel mill and 1 kilometer south of the SCAQMD monitoring trailer at Foothill Blvd. and Colorado Avenue. Redwood School was so close to the steel mill that particulate matter (shiny flakes, possibly from slag waste) could be seen in the air and on the ground.

Two urban centers were chosen for sampling sites. These are different from those mentioned in the original proposal for the following reasons. The southcentral Los Angeles area, noted for high incidences of lung cancer (Henderson et al., 1975), was close to and essentially the same as the Long Beach site previously described. West Los Angeles, where high levels of oxides of nitrogen have been measured (California Air Resources Board), is very close to the downwind freeway location. The Santa Ana Canyon was ruled out because of uncertainty as to the origin and trajectory of the air

parcel in the canyon. One urban site chosen (#4) was at the California State University, Los Angeles (CSULA). The campus is immediately downwind from the most concentrated urban area in the SCAB, downtown Los Angeles. This is also where a longpath infrared (LPIR) spectrometer and other air monitoring instrumentation will be set up shortly by SAPRC. The hi-vols were placed on the west roof of the Physical Science Building, nine stories above ground level. Although there are no SCAQMD stations nearby, CSULA is about 7 miles west of the downtown Los Angeles monitoring site.

The other urban sampling site (#6) was at Harvey Mudd College in Claremont. This was chosen because it represented an intermediate receptor area, often had high ozone levels, and SAPRC was already thoroughly monitoring air pollutants at this location, both with LPIR and conventional instruments. The hi-vols were placed on the roof of the Jacob Science Center, about 10 meters above the ground.

Finally, Riverside remained the smog receptor site (#8). The hi-vols were mounted on the ARB Mobile Laboratory for Air Pollution Research location just north of Fawcett Laboratory at SAPRC. This facility is a fully instrumented air monitoring station.

2. Collection Protocol. The Teflon-impregnated glass fiber hi-vol filters (TIGF) used in this study were pre-cleaned by washing the filters in methanol and dichloromethane followed by vacuum drying at 80°. The filters were then numbered, weighed to 0.1 mg at 50% RH and 75°F, placed in file folders and then envelopes.

The hi-vol samplers were set up at each site about one meter apart. The exhaust from the blower motors was carried several meters away by 4-inch plastic ducting. The flow rates were adjusted to 40 SCFM by an orifice plate flow calibrator (Sierra Instruments, Model 330). Filters were changed at 9:00 a.m. and 9:00 p.m. PDT; since one person was assigned two sites the actual time varied  $\pm$  1/2 hour. Two three-day sampling episodes were conducted, July 11-13, 1979 and July 24-26, 1979. During the latter period washed and fired glass fiber filters were used. This set was held in reserve. Immediately after the second collection the calibration of all hi-vols was checked to verify flow stability.

Upon removal from the sampler, filters were wrapped with solvent-washed aluminum foil, placed back in file folders and envelopes, sealed

in plastic bags, and placed in a cooler with dry ice. Upon arrival at SAPRC one filter from each site was immediately transferred to storage at  $-20^{\circ}\text{C}$ . The other was equilibrated for 12 hours at 50% RH and  $75^{\circ}\text{F}$ , weighed, rewrapped in foil, folder, envelope, and plastic bag, and placed in the freezer prior to solvent extraction.

3. Extraction and Sample Handling. The particulate-loaded filters were removed from storage, and each set was extracted with a 1:1:1 mixture of methanol, toluene, and dichloromethane (150 ml/filter) by ultrasonication under a nitrogen atmosphere. All solvents used were low residue, "distilled in glass" grade, and all sample handling was carried out under red light. The disintegrated filter material and other insoluble matter was removed by filtration through a prewashed 0.45 micron Teflon membrane filter, which was then rinsed three times with more of the solvent mixture. The extract solution was then reduced in volume in vacuo ( $35^{\circ}\text{C}$ ,  $>20$  torr.); the residue was transferred to tared, Teflon-sealed vials; the residual solvent was removed with a stream of dry nitrogen at  $35^{\circ}\text{C}$  until constant weight ( $\pm 1\%$ ) was achieved; and, the mass of the extracted material was recorded. The residue was then dissolved in helium-degassed dimethylsulfoxide (Mallinkrodt spectraAR grade) and delivered to the microbiology laboratory, where the samples were quick-frozen by immersion in liquid nitrogen and stored as solid solutions prior to Ames testing. Sixteen such samples were prepared, each consisting of the combined extract of three 12-hour particulate samples collected during the 12-hour "day" and "night" periods of three consecutive days at the eight sites, with one exception; a power failure at the West Los Angeles site (west of I-405) during the final collection period limited the "night" sample to two filters.

4. Mutagenic Assay. Preliminary testing was carried out on the 16 samples in three separate screens. Samples were plated in duplicate with five strains, TA-1535, 1537, 1538, 98 and 100, at concentration levels of 1, 10 and 100  $\mu\text{g}/\text{plate}$ , both without metabolic activation and with 2% and 10% (liver v/v mix) S9 (Aroclor-1254 induced rat liver homogenate) in the top agar. 0.5 ml of 0.667 M sodium phosphate buffer solution was added to each sample tested for direct activity in order to maintain volume and pH consistency with those plated with S9. Standard recommended positive

control mutagens (de Serres and Shelby, 1979a,b) were used to determine and monitor strain response. All samples were handled under inactinic light. Test plates were incubated for 63 hours and revertant colonies were counted shortly thereafter.

The preliminary data were used to determine the most responsive strains, and optimum sample and S9 concentrations for use in the quantitative determination of mutagenic potency. Strains TA1538 and TA98 displayed a significant mutagenic response to all samples tested. Highest reversion frequencies were observed with and without 2% S9, with a 10% concentration leading to a diminished response.

Quantitative testing of all 16 samples was performed concurrently, with each sample plated in triplicate with TA98 at levels of 10, 20, 40, 60, 80, 100, 200, and 400  $\mu\text{g}$  extract/plate, with and without 2% S9. Dose-response relationships of the standard control mutagens 2-nitrofluorene and benzo(a)pyrene were determined in parallel with the ambient samples to determine the relative strain sensitivity and to allow some comparison of the ambient samples' relative activity. The tester strain was also checked for resistance to ampicillin and sensitivity to ultraviolet and crystal violet to ensure the proper genotype. Test plates were treated in the same manner as in the preliminary screen, and a random sampling of them was checked for true reversion after incubation. The slopes of the sample dose responses were determined in revertants/ $\mu\text{g}$  extract by application of a linear least squares program to the means of triplicate plates at each dose level. Data points which showed a departure from linearity at high doses were rejected. Gravimetric and mutagenicity data for the 16 samples are presented in Table 9. All solvent and filter blanks were non-mutagenic; specific activities for the standards benzo(a)pyrene and 2-nitrofluorene were 361 and 475 rev/ $\mu\text{g}$ , respectively.

### Results and Discussion

The reproducibility of our mutagenic assay techniques has been discussed in section V, and the associated random error has been determined to be about  $\pm 15\%$ .

We are continuing experiments aimed toward determining this figure more precisely and establishing firm confidence limits for our data.

Table 9. Gravimetric and mutagenic data of samples collected for the geographical distribution study,  
July 11-14, 1979

Site	Sample No.		Collection Time	TSP (mg)	Extract (mg, % TSP)	Specific Activity (rev/ $\mu$ g extract on TA98)	
	Day/Night	Time				-S9	+2% S9
West Los Angeles	1D		30:57	213.8	68.69(32.1)	0.50	0.60
	1N		25:46	164.6	61.19(37.2)	0.40	0.54
Westwood	2D		32:11	287.2	79.50(27.7)	0.94	1.7
	2N		37:25	247.9	87.00(35.1)	1.0	1.2
Long Beach	3D		32:29	192.3	48.86(25.4)	1.2	2.4
	3N		38:01	164.4	64.16(39.0)	0.32	0.46
Cal State Los Angeles	4D		37:45	429.1	118.97(27.7)	0.50	1.3
	4N		32:00	227.4	79.39(34.9)	0.74	0.78
Costa Mesa	5D		34:12	181.8	36.11(19.9)	0.6	0.84
	5N		35:16	148.7	65.81(44.2)	0.37	0.35
Claremont	6D		35:33	393.3	143.98(36.6)	0.38	1.75
	6N		32:06	274.2	101.30(36.9)	0.97	1.2
Fontana	7D		34:18	877.8	195.00(22.2)	0.49	0.69
	7N		35:16	532.7	140.48(26.4)	0.59	0.98
Riverside	8D		32:42	477.5	183.21(38.4)	0.15	0.29
	8N		35:16	340.7	124.29(36.5)	0.68	1.0

We believe currently that systematic error resulting from incomplete extraction of organics from the collected particulate is less than 5% for direct-acting mutagens and less than 7% for activatable mutagens. This estimate is derived from the "worst case" extraction efficiency experiment using a heavily loaded glass-fiber filter and from the fact that extraction efficiency for TIGF filters is consistently higher than that for ordinary glass fiber filters (Table 3). At the present time, then, we conservatively assign an error to the entire process of analysis of approximately  $\pm 20\%$  for the assay of direct activity, and a slightly larger uncertainty in the determination of activatable mutagenicity, as indicated by the response with strain TA98.

The sampling period used, 12 hours, represents a compromise of the ideal of instantaneous and continuous monitoring, as is possible for many pollutants, due to the sample size requirements of the Ames assay and the desire to minimize differences in processing history among the sample. We believe, however, that 12-hour samples are superior to longer-term collections in terms of fidelity to real world conditions. We are currently investigating (under EPA funding) the possible benefits (apart from improved time resolution) to be gained from shorter sampling periods.

The experimentally measured variables, presented in Table 9, include the total collection time for each sample (the sum of the exact durations of the three nominal 12-hour sampling periods), the mass of each collected particulate sample after equilibration at 24°C and 50% relative humidity, the mass of each extract after removal of solvent, and the specific mutagenic activity, in revertants/ $\mu\text{g}$ , of each extract, both with and without metabolic activation. No correlation among any of these variables is evident. Specific activity values, which should reflect the concentration of mutagenic material in the extracts, vary over nearly an order of magnitude, indicating substantial variation in extract composition among the sixteen samples. During the day (9 am-9 pm) sampling periods, the extract from the Long Beach sample showed the highest specific activity, at 1.2 and 2.4 revertants/ $\mu\text{g}$  (-S9/+S9). Among the nighttime samples, the Westwood and Claremont extracts were most active.

Table 10 presents several calculated values derived from the raw data. The sampled volume was obtained from the measured collection time

Table 10. Calculated mutagenic values of samples collected for the geographical distribution study, July 11-14, 1979.

Site	Sample No. Day/Night	Sampled Volume (m <sup>3</sup> )	Total Activity (in thousands) (Rev/36-hr sample)		Mutagen Density (Rev/m <sup>3</sup> )		Mutagen Loading (Rev/mg particulate)	
			-S9	+S9(2%)	-S9	+S9(2%)	-S9	+S9(2%)
West Los Angeles	1 D	2104	34	41	16	20	160	190
	1 N	1751	24	33	14	19	140	200
Westwood	2 D	2188	75	135	34	62	260	470
	2 N	2543	87	104	34	41	350	420
Long Beach	3 D	2207	59	117	27	53	300	610
	3 N	2584	21	30	7.9	11	120	180
Cal State Los Angeles	4 D	2566	59	155	23	60	140	360
	4 N	2175	59	62	27	28	260	270
Costa Mesa	5 D	2325	24	30	10	13	130	170
	5 N	2397	24	23	10	9.6	160	150
Claremont	6 D	2416	55	108	23	45	140	270
	6 N	2182	98	122	45	56	360	440
Fontana	7 D	2331	96	135	41	58	110	150
	7 N	2397	83	138	34	57	150	260
Riverside	8 D	2223	27	53	12	24	58	110
	8 N	2397	85	124	35	52	250	360

and the calibrated flow rate of the samplers (40 c.f.m.). The total mutagenic activity of each extract, in thousands of revertants, was obtained by multiplying the extract mass by its specific activity. The average mutagen density during each sampling period was calculated by dividing the total activity by the sampled volume, and is expressed as revertants/cubic meter. This parameter corresponds directly with the dose of mutagenic material, as defined by TA98, available for inhalation by the human population. Although the upper effective mean aerodynamic diameter cut-off employed in our samplers (100  $\mu\text{m}$ ) results in the inclusion of some non-inhalable particulate in the samples, the size distribution of particles bearing mutagenic materials (Miguel, 1979; Miguel and Friedlander, 1978; Kertesz-Saringer et al., 1971; Pierce and Katz, 1975) indicates that these mutagen density values should correspond to the average inhalable dose. The final calculated parameter in Table 10, mutagen loading, is obtained by division of the total mutagenic activity of each sample by the mass of the collected particulate. This value, expressed as revertants/milligram particulate, represents the concentration of mutagenic material in the sampled particulate on a mass basis. This value is intended to reflect the mutagen loading of the particulate independent of its number density, and might be useful in an attempt to identify areas which are exposed to particularly "high potency" particulate, resulting from either local emissions or chemical modification during transport. Such an analysis would ideally include only particles in the respirable size range, and the inclusion of larger particles in our samples allows the size distribution of the sample to affect this value as well.

Figures 19 and 20 present the data of Table 10 in graphical form. Figure 19 (Airborne Particulate Mutagen Density) reveals large differences in the intensity and diurnal variation of the activity at the eight sites. While we have not yet completed analysis of this data, some preliminary conclusions can be drawn:

- The West Los Angeles and Costa Mesa air (sites 1 and 5) showed low levels of activity which did not exhibit a significant diurnal variation. This would seem to indicate that the Costa Mesa site was not strongly influenced by the proximate power plant during the sampling period. In contrast, the Fontana site (site 7) (near a steel mill) apparently received a high mutagen dose with no significant diurnal variation.

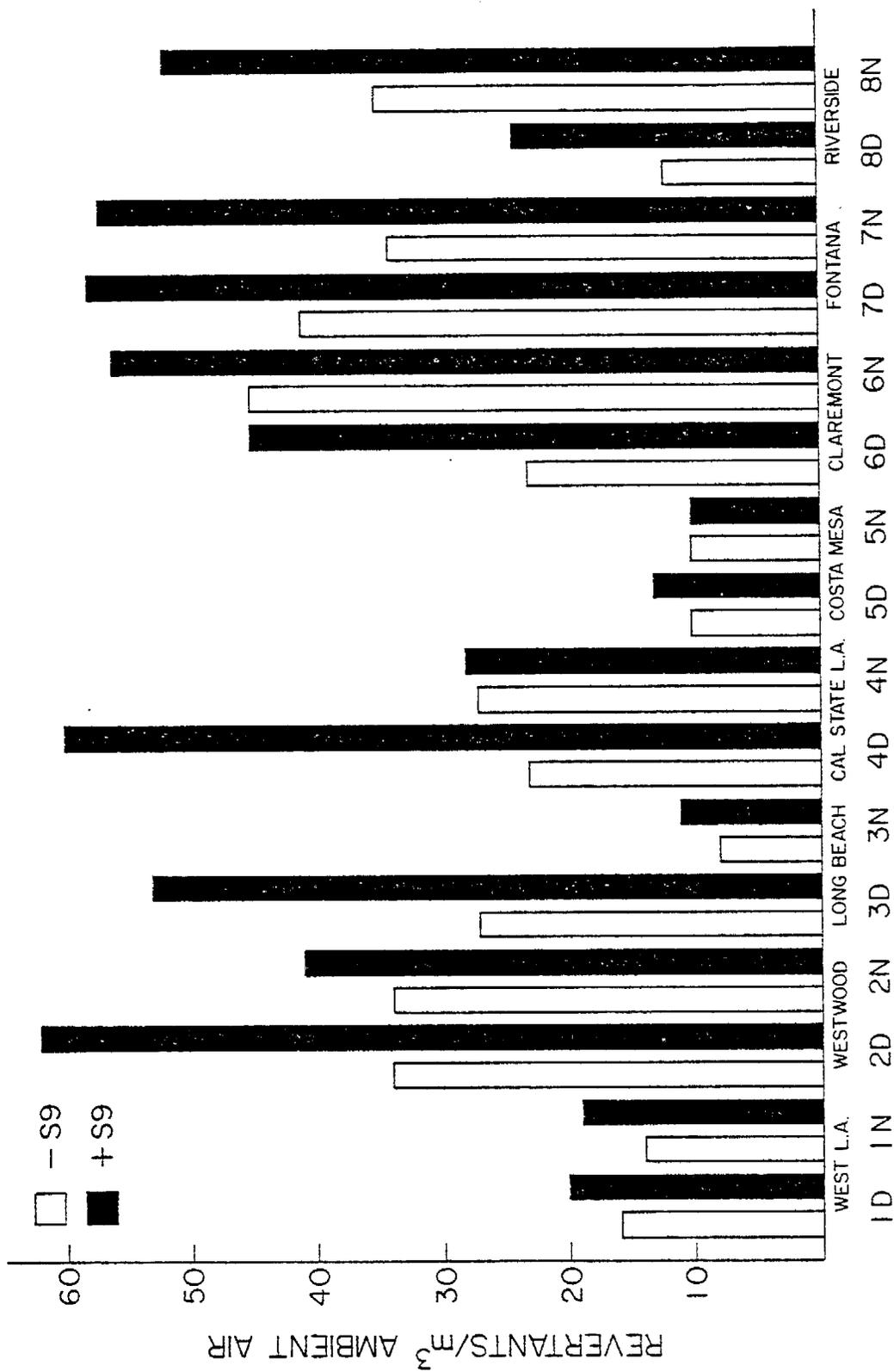


Figure 19. Airborne particulate mutagen density (revertants/m<sup>3</sup> ambient air) using strain TA98 of total extracts of samples collected at eight sites throughout the South Coast Air Basin, July 11-14, 1979.

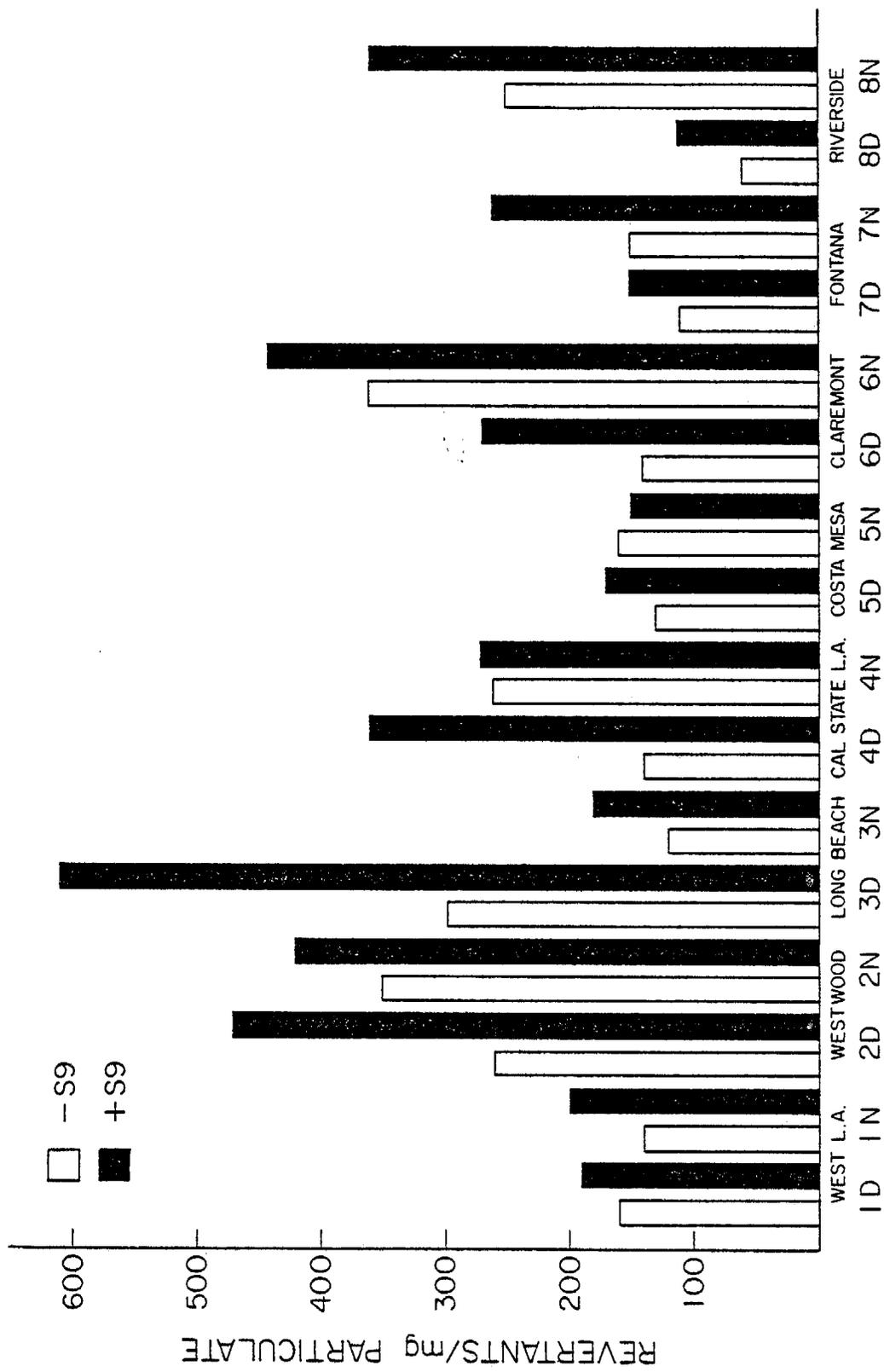


Figure 20. Airborne particulate mutagen loadings (revertants/ $\mu\text{g}$  particulate) determined using strain TA98 of total extracts of samples collected at eight sites throughout the South Coast Air Basin, July 11-14, 1979.

- The five remaining sites can be divided into two groups based on the observed diurnal variation in measured activity; the Westwood, Long Beach and Downtown locations (sites 2, 3, and 4) show a decrease in mutagen density (Figure 19) at night, while the Claremont and Riverside samples displayed the opposite behavior. The mutagen loading parameters (Figure 20) show the same general trend. This behavior seems most likely to reflect a transport phenomenon; the meteorological data which we have compiled is consistent with this hypothesis.

- Comparison of the mutagen loading values for the first subgroup (sites 2, 3, and 4) reveals an interesting difference among the three areas. The Westwood samples can be taken to represent vehicular emissions almost exclusively, as shown by the difference in activity measured on either side of I-405 (sites 1 and 2). The diurnal behavior of the Cal State Los Angeles (site 4) mutagen loading figures parallels closely that of the samples taken at Westwood, suggesting that this area, too, is dominated by vehicular emissions. The Long Beach samples, however, differ from the other two areas in the diurnal variation of mutagen loading. This difference may reflect the impact of non-vehicular (industrial) emission sources in the Long Beach area.

- Among the five sampling areas discussed above, there is a consistent trend observable in the ratio of direct to activatable mutagenicity as a function of time of day. The relationship is observable in terms of both mutagen density and mutagen loading in that this ratio increases at night.

The values of several air quality parameters were compiled during the collection. Presented in Table 11 are both the average pollutant gas levels and the average peak one-hour levels reached by these parameters during the collection periods. A trajectory study was also carried out in order to determine general transport behavior during the collections. Back trajectories were calculated for each sampling site (Figures 21 and 22) using wind speed and direction data from about 60 of 80 possible stations in the South Coast Air Basin. These trajectories were derived in the following way:

Table 11. Air quality parameters for the geographical distribution study sites: July 11 (9 am) to July 14 (9 pm), 1979.

Site	ARB Designator	Ozone		Nitric Oxide		Nitrogen Dioxide		Sulfur Dioxide		Carbon Monoxide		
		pphm		pphm		pphm		pphm		pphm		
		Mean	Peak Ave.**	Mean	Peak Ave.**	Mean	Peak Ave.**	Mean	Peak Ave.**	Mean	Peak Ave.**	
West L.A./ Westwood	70-00086	1D/2D	5.2	9.0	0.7	2.3	3.8	8.0	0.6	1.3	2.1	3.3
		IN/2N	0.7	2.3	4.2	7.3	5.1	7.7	0.8	1.0	3.2	4.7
Long Beach	70-00072	3 D	1.9	3.7	ND**	ND	ND	ND	0.4	1.0	2.5	3.7
		3 N	1.1	2.3	0.6	2.5	2.3	3.3	0.3	1.3	1.6	2.7
Downtown Los Angeles	70-00001	4 D	6.1	9.8	0.8	2.0	6.4	10.7	1.1	2.0	2.7	3.7
		4 N	1.5	3.0	2.7	9.0	4.9	8.0	0.9	2.0	2.3	3.7
Costa Mesa	30-00194	5 D	2.2	3.3	0.2	0.7	1.2	2.3	0.3	1.0	1.2	1.7
		5 N	2.3	3.0	0.4	1.3	0.3	1.0	0	0	1.1	1.7
Claremont	---	6 D	9.8	16.3	0.7	2.0	6.8	9.1	ND	ND	ND	ND
		6 N	0.3	2.3	4.0	6.4	6.9	11.1	ND	ND	ND	ND
Fontana	36-00176	7 D	14.4	27.3	0.8	3.0	5.9	10.3	3.0	5.0	2.2	2.3
		7 N	2.9	8.0	1.8	4.3	6.5	8.0	0.5	2.3	2.1	3.0
Riverside	33-00144	8 D	12.4	20.3	0.7	1.0	2.5	3.3	2.2	3.0	3.9	5.7
		8 N	1.1	6.3	2.9	7.0	4.1	5.3	0.3	1.3	4.3	5.7

\*Refers to the mean of the peak hourly measurements during each sampling period

\*\*ND = No Data

Values obtained from only two days' measurements.

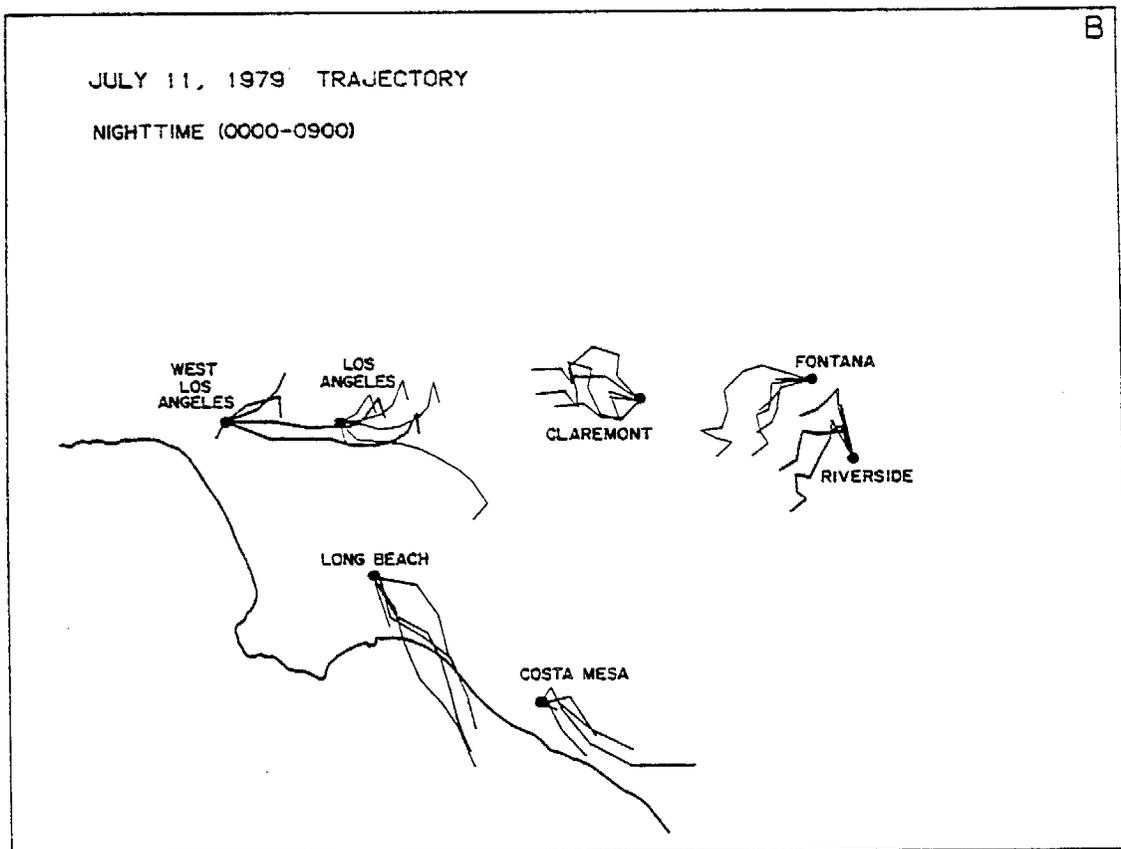
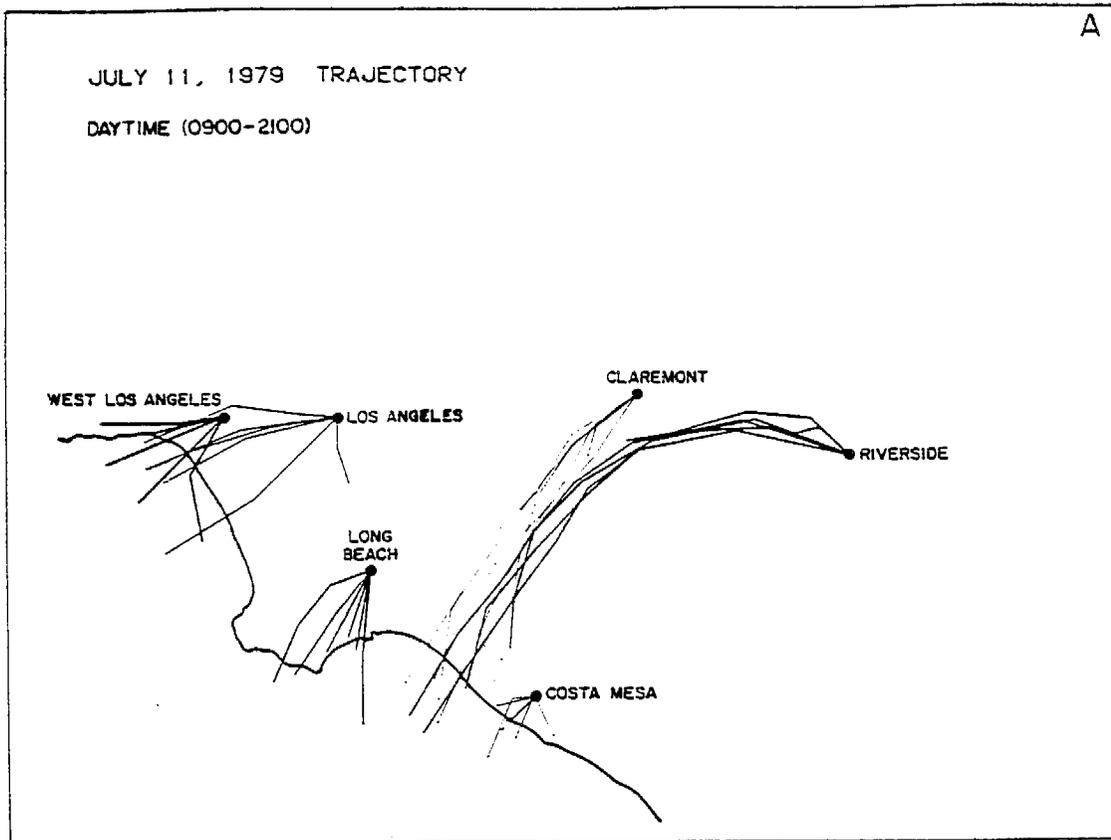


Figure 21. Backwind trajectories calculated for the eight sampling sites throughout the South Coast Air Basin on July 11, 1979. (A) Daytime trajectory. (B) Nighttime trajectory.

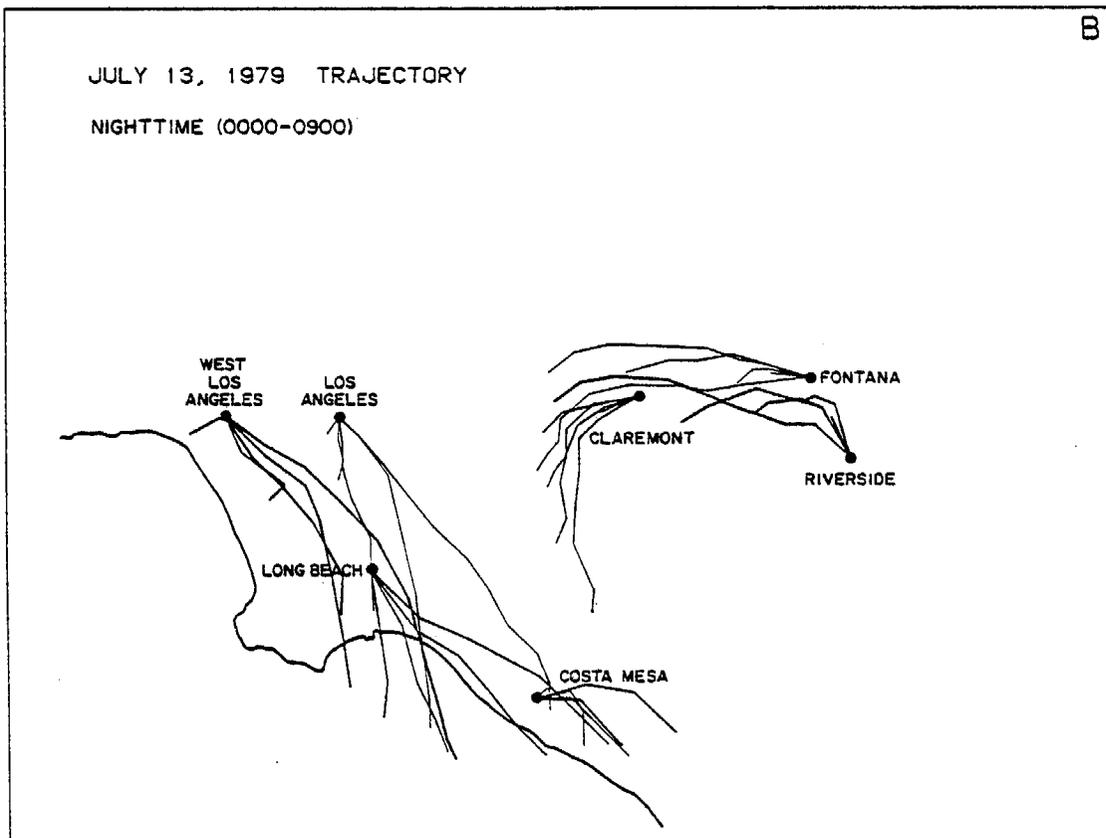
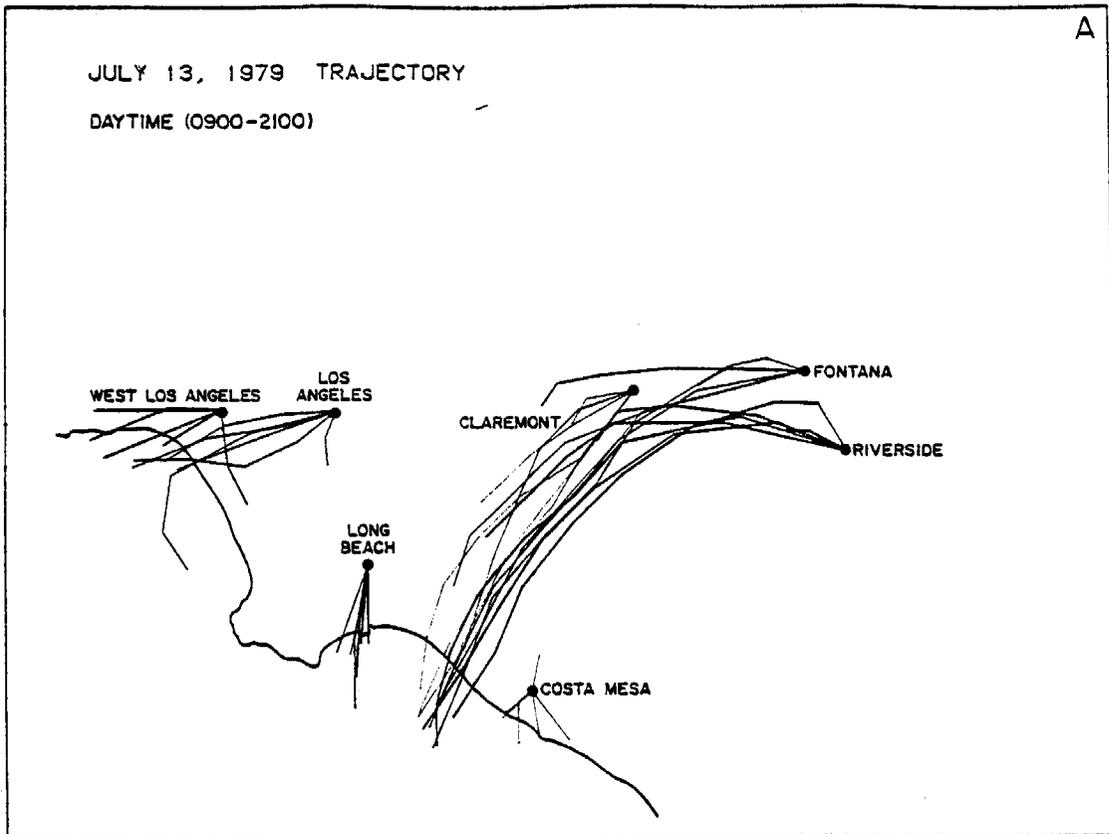


Figure 22. Backwind trajectories calculated for the eight sampling sites throughout the South Coast Air Basin on July 13, 1979. (A) Daytime trajectory. (B) Nighttime trajectory.

(1) The sampling interval was divided into two-hour segments.

(2) For each segment the wind speed and direction data for the previous hour at the three closest monitoring stations was used to calculate a vector, with the closer stations receiving a proportionately higher mathematical weighting.

(3) This vector was plotted, corresponding roughly to where the air parcel originated during the middle of the segment.

(4) This process was repeated for the next previous hour using another set of three closest stations.

(5) Vectors were backplotted to the start of the collection time or until no station was deemed close or a mountain range was reached.

The days ended at 2400 hours because of the way the data was handled. Thus, for the night sample two plots were made; one for the period before midnight, and one for the period after. A typical trajectory plot is shown in Figure 22a for the daytime collection on July 13, 1980. Each site has six trajectories emanating from it, corresponding to plots started at the times of 1100, 1300, 1500, 1700, 1900 and 2100 hours. The 1100 hour plot would be the shortest since vectors are calculated at 1000 and 0900 hours.

The trajectories for July 11 and 13 are shown in Figures 21 and 22. The period from 2100 to 2400 is omitted because there are only two short trajectories on it. The daytime trajectories are quite similar, showing the strong onshore flow normal during this time of year. The first night shows light and somewhat variable winds and coastal areas having mild offshore breezes. The night of July 13 indicates relatively strong winds from the south and curving inland, apparently due to the influence of the San Gabriel Mountains to the north.

## VIII. AMES MUTAGENICITY ASSAY OF GAS-PHASE SAMPLES

Introduction. To evaluate the methods of testing gas-phase samples for mutagenicity as a means of minimizing potential filter artifacts, we proposed to apply the Ames Salmonella/mammalian-microsome mutagenicity test for screening ambient gases. The determination of mutagenicity of ambient pollutants with the Ames test is seriously hampered by their low concentration, which is below the tester strain threshold of detection for all but the most potent mutagens (Ames et al., 1975; Bartsch et al., 1975). The problem of low concentration might be circumvented by various enrichment techniques. The choice of an appropriate technique for concentrating unknown products is, at best, an educated guess. Nevertheless, in our view, no other procedure appeared as promising, and our efforts were directed towards concentrating and collecting gaseous or vapor-phase materials using several techniques.

Experimental. The first method investigated was the use of a solid absorbent (Tenax GC) to collect ambient gases. This was thought to deliver adequate quantities of material for Ames test screening when used with the Teflon outdoor-chamber smog experiments. Our experience with this method involved the exposure of dimethylethanolamine (DMEA) with pollutant gases. Eluted samples were tested with strains TA98 and TA100 with and without metabolic activation (2% S9 liver v/v mix).

Collection of gases was also investigated in the DMEA study by passing them through a cold trap cooled to liquid argon temperature (-185.76°C). Most of the material collected was water vapor, plus water soluble gases and vapors. The samples were tested with strain TA98 with and without metabolic activation (2% S9 liver v/v mix). Finally, direct exposure of the test bacteria on petri plates to flowing filtered gases was explored during the DMAE study. Five tester strains, TA1535, TA1537, TA1538, TA98 and TA100, and two levels of metabolic activation (2% and 10% S9 liver v/v mix) were employed to screen for direct mutagenicity and possible activation. Petri plates were housed in sealed Billup-Rothenberg chambers uncovered for maximum gas-agar exchange. Gases were pumped out of the outdoor smog chamber

three Billup-Rothenberg chambers at a flow rate of approximately 1 liter per minute for eight hours at room temperature.

Results and Discussion. No significant mutagenic activity was detected with any of these collection methods. Cold-trap collection is likely to be unsuitable due to the presence of potentially biologically contaminating substances, dilute sample concentration and osmotic effects from large amounts of water. No further investigation is planned with this technique. Direct exposure of the tester strains on petri plates to air, filtered gases also did not provide any significant activity. It might be thought that the presence of sub-ppm amounts of ambient pollutants might be below the sensitivity threshold for detection of the tester strains.

A concentration procedure such as collection with the Tenax cartridge is likely to produce artifact errors. Therefore, the sampling would probably have to be done following diffusion-denuding and filtering to help minimize artifact chemical reactions on the solid interface. When we intend to continue to explore enrichment techniques, we suggest that these lines of investigation should also be given reduced priority.

## IX. REFERENCES

- Ames, B. N. (1979): Identifying environmental chemicals causing mutations and cancer. *Science*, 204, 587-573.
- Ames, B. N. and McCann, J. (1976): Carcinogens are mutagens: a simple test system. In Screening Tests in Chemical Carcinogens, IARC Scientific Publication #12, Montesaro, R., Bartsch, H., Tomatis, L. (eds.). Lyon, France, International Agency for Research on Cancer.
- Ames, B. N., Durston, W. E., Yamasaki, E. and Lee, F. D. (1973): Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. *Proc. Natl. Acad. Sci.*, 70, 2281-2285.
- Ames, B. N., McCann, J. and Yamasaki, E. (1975): Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. *Mutat. Res.*, 31, 347-364.
- Bartsch, H., Malaveille, C. and Montesano, R. (1975): Human, rat and mouse liver mediated mutagenicity of vinyl chloride in *S. typhimurium* strains. *Int. J. Cancer*, 15, 429-437.
- Belser, W. L., Jr., Shaffer, S. D., Bliss, R. D., Hynds, P. M., Yamamoto, L., Pitts, J. N., Jr. and Winer, J. A. (1980): A standardized procedure for quantification of the Ames Salmonella/mammalian-microsome mutagenicity test. Environ. Mut., in press.
- Blot, W. J., Brinton, L. A., Fraumeni, J. F., Jr. and Stone, B. J. (1977): Cancer mortality in U. S. counties with petroleum industries. *Science*, 198, 51-53.
- Brockhaus, A. (1974): Sampling of tetracyclic aromatic hydrocarbons under atmospheric conditions. *Atmos. Environ.*, 8, 521.
- Brusick, D. L. (1978): Automated plate-counting assays in the Ames Salmonella/microsome mutagenicity test. *Pharmaceutical Technol.*, 2, 37-40, 65.
- California Air Resources Board: California Air Quality Data. Quarterly Reports and Ten Year Summary, 1963-1978.

- Chriswell, C., Glatz, B. A., Svec, H. J. and Fritz, J. S. (1978): Mutagenic analysis of drinking water samples. Presented at the Environmental Protection Agency Symposium on the "Application of Short-Term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures," Williamsburg, VA, February 21-23, 1978.
- Davis, S. M. and Lunsford, J. H. (1978): Surface reactions of SO<sub>2</sub> and NO<sub>2</sub> on hydrated silica and silica-alumina. *J. Coll. Interf. Science*, 65, 352-364.
- De Flora, S. (1978): Metabolic deactivation of mutagens in the Salmonella/microsome test. *Nature*, 271, 455-456.
- De Serres, F. J. and Shelby, M. D. (1979a): The Salmonella mutagenicity assay: Recommendations. *Science*, 203, 563-565.
- De Serres, F. J. and Shelby, M. D. (1979b): Recommendations on data production and analysis using the Salmonella/microsome mutagenicity assay. *Mutat. Res.*, 64, 159-165.
- Dickson, J. (1978): Personal communication.
- Eisenstadt, E. (1978): Personal communication.
- Epler, J. L., Young, J. A., Hardigree, A. A., Rao, T. K., Guerin, M. R., Rubin, I. B., Ho, C. H. and Clark, B. R. (1978): Analytical and biological analyses of test material from the synthetic fuel technologies. I. Mutagenicity of crude oils determined by the Salmonella typhimurium/microsome activation system. *Mutat. Res.*, 57, 265-276.
- Fisch, J. E. and Beck, D. J. (1978): Mutagenicity of platinum coordination complexes in Salmonella typhimurium. Presented at the Annual Meeting of the American Society of Microbiology, Las Vegas, NV, May 14-19, 1978.
- Grosjean, D. (1975): Solvent extraction and organic carbon determination in atmospheric particulate matter: the organic extract-organic carbon analyzer (OE-OCA) technique. *Anal. Chem.*, 47, 797-805.
- Henderson, B. R., Gordon, R. J., Menck, H., Soohoo, J., Martin, S. P. and Pike, M. C. (1975): Lung cancer and air pollution in southcentral Los Angeles county. *Amer. J. Epidem.*, 101, 477-488.

- Hueper, W. C., Kotin, P., Tabor, E. C., Payne, W. W., Falk, H. L. and Sawicki, E. (1962): Carcinogenic bioassays on air pollutants. Arch. Pathol., 74, 89-116.
- Jones, P. W., Glammar, R. D., Strup, P. E. and Standford, T. B. (1976): Efficient collection of polycyclic organic compounds from combustion effluents. Environ. Sci. Technol., 10, 806-810.
- Kertesz-Saringer, M., Meszaros, E. and Varkonyi, T. (1971): On the size distribution of benzo(a)pyrene containing particles in urban air. Atmos. Environ., 5, 429-431.
- McMahon, R. E., Cline, J. C. and Thompson, C. Z. (1979): Assay of 855 test chemicals in ten tester strains using a new modification of the Ames test for bacterial mutagens. Cancer Res., 39, 682-693.
- Miguel, A. H. (1979): Distribution of polycyclic aromatic hydrocarbons with respect to particle size in Pasadena aerosols in the submicrometer range. In: Polynuclear Aromatic Hydrocarbons (P. W. Jones and P. Leber, eds.), Ann Arbor Science Pubs., Ann Arbor, MI.
- Miguel, A. H. and Friedlander, S. K. (1978): Distribution of benzo(a)pyrene and coronene with respect to particle size in Pasadena aerosols in the submicron range. Atmos. Environ., 12, 2407-2413.
- New Brunswick Scientific Co. (1979): Guide to counting colonies with an automated instrument. Available upon request from New Brunswick Scientific Co.
- Peters, J. and Siefert, B. (1980): Losses of benzo(a)pyrene under the conditions of high-volume sampling. Atmos. Environ., 14, 117-119.
- Pierce, R. C. and Katz, M. (1975): Dependency of polynuclear aromatic hydrocarbon content on size distribution of atmospheric aerosols. Environ. Sci. Technol., 9, 347-353.
- Pitts, J. N., Jr. (1979): Photochemical and biological implications of the atmospheric reactions of amines and benzo(a)pyrene. Presented to the Royal Society of London at a discussion entitled "Pathways of Pollutants in the Atmosphere," London, November 3-4, 1977. Phil. Trans. R. Soc. Lond. A, 290, 551-576.

- Pitts, J. N., Jr., Van Cauwenberghe, K., Grosjean, D., Schmid, J. P.,  
Fitz, D. R., Belser, W. L., Jr., Knudson, G. B. and Hynds, P. M.  
(1978): Atmospheric reactions of polycyclic aromatic hydrocarbons.  
Facile formation of mutagenic nitroderivatives. *Science*, 202, 515-519.
- Pupp, C., Lao, R. C., Murray, J. J. and Pottie, R. F. (1974): Equili-  
brium vapor concentrations of some polycyclic aromatic hydrocarbons,  
As<sub>4</sub>O<sub>6</sub> and SeO<sub>2</sub> and the collection efficiencies of these air  
pollutants. *Atmos. Environ.*, 8, 915-925.
- Rondia, D. (1965): Sur la volatilité des hydrocarbures polycycliques.  
*Int. J. Air Water Pollut.*, 9, 113-121.
- Stokes, J. L. and Bayne, H. G. (1957): Growth rates of Salmonella  
colonies. *J. Bact.*, 74, 200-206.
- Tomingas, R. (1979): Remarks on the sampling procedure for polycyclic  
aromatic hydrocarbons from the atmosphere. *Fresenius Z. Anal. Chem.*  
297, 97-101.
- Van Cauwenberghe, K. A., Van Vaeck, L. and Pitts, J. N., Jr. (1980):  
Chemical transformations of benzo(a)pyrene on filters by ambient  
levels in air. To be submitted for publication.

X. LIST OF PUBLICATIONS FROM SAPRC-ARB MUTAGEN PROGRAM

Chemical and Microbiological Studies of Mutagenic Pollutants in Real and Simulated Atmospheres

Proceedings from the First EPA Symposium on Application of Short-Term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures

J. N. Pitts, Jr., K. A. Van Cauwenberghe, D. Grosjean, J. P. Schmid, D. R. Fitz, W. L. Belser, Jr., G. B. Knudson, and P. M. Hynds  
Williamsburg, Virginia; February 21-23, 1978.

Interactions Between Diesel Emissions and Gaseous Co-Pollutants and Photochemical Air Pollution: Some Health Implications

Proceedings from the EPA Symposium on Health Effects of Diesel Engine Emissions

J. N. Pitts, Jr., A. M. Winer, D. M. Lokensgard, S. D. Shaffer, E. C. Tuazon, and G. W. Harris  
Cincinnati, Ohio; December 3-5, 1979

"Atmospheric" Epoxidation of Benzo(a)pyrene by Ozone: Formation of the Metabolite Benzo(a)pyrene-4,5-Oxide

Science (in press)

J. N. Pitts, Jr., D. M. Lokensgard, P. S. Ripley, K. A. Van Cauwenberghe, Luk Van Vaeck, S. D. Shaffer, A. J. Thill, and W. L. Besler, Jr.

A Standardized Procedure for Quantification of the Ames Salmonella/Mammalian-Microsome Mutagenicity Test

Environmental Mutagenesis (in press)

W. L. Belser, Jr., S. D. Shaffer, R. D. Bliss, P. M. Hynds, L. Yamamoto, J. N. Pitts, Jr., and J. A. Winer



## Appendix A. Protocol for Extraction and Acid/Base Separation of POM

### Solvents

1:1:1 toluene/dichlorometane/methanol ("super solvent"), distilled in glass, saturated with nitrogen, freshly mixed for each day's work; diethyl ether, distilled in glass, 2% ethanol, stored under N<sub>2</sub>.

### Glassware

Clean, rinsed with solvent prior to use.

### Stock Solutions

6N HCl, 2N HCl (organic-free), 5% aqueous KHC<sub>3</sub>, saturated aqueous NaCl (brine), 6N NaOH; the water used for these preparations is doubly distilled.

### Filter Extraction

This procedure is to be performed under red light. Unexposed edges of each filter are trimmed, the filter is cut into ~1 cm squares with scissors or a pizza cutter (cleaned and rinsed with super solvent) and transferred to a glass-stoppered Erlenmeyer flask. Super solvent is added (150 ml/8" x 10" filter), the flask is flushed with N<sub>2</sub>, stoppered, and placed in an ultrasonic bath for 30 minutes. The mixture is vacuum filtered and the filter is washed 3 times with 25 ml of super solvent. A portion of this total extract can be used for mutagenic testing after filtration through a 0.45 micron millipore Teflon filter.

### Acid/Base Separation of Ether-Soluble Organics (1-4 filters)

The total extract is transferred to a round-bottomed flask, 0.5 g. KHC<sub>3</sub> is added and the solution is reduced in volume in vacuo until aspirator pressure is reached (12-20 Torr). Diethyl ether (peroxide-free) is added to the flask (50 ml/filter) followed by 25 ml 5% KHC<sub>3</sub> solution. The mixture is swirled to dissolve the residue and transferred to a separatory funnel.

1. Extraction of Sulfonic and Carboxylic Acids. The mixture is shaken, the lower layer is withdrawn, and its pH is checked. If the pH is less than 8, the ether layer is extracted with further 25 ml

portions of 5%  $\text{KHC}\text{O}_3$  solution until this value is obtained. The aqueous layers are combined and extracted 2 times with 25 ml of ether, which is added to the original ether layer. The aqueous layers are set aside, labeled "acids".

2. Extraction of Bases. The ether layer is extracted with 25 ml portions of 2N HCl until the pH of the aqueous layer is 1. The aqueous layers are combined and extracted 2 times with 25 ml of ether, which is added to the ether layer. The aqueous layer is set aside, labeled "bases".

3. Recovery of the Neutral Fraction. The ether layer is shaken with 25 ml brine, the lower layer is discarded, and the ether layer is transferred to a stoppered flask containing 1 g anhydrous sodium sulfate. The solution is allowed to stand for 30 minutes, with occasional swirling. The solution is then filtered through an 0.45 micron Millipore Teflon filter, reduced in volume in vacuo, and transferred to a tared vial. The remaining solvent is removed with a stream of  $\text{N}_2$  to constant weight and the weight of the residue is recorded.

4. Recovery of the Acidic Fraction. The basic aqueous solution labeled "acids" is transferred to an appropriately sized beaker containing a Teflon-coated magnetic stirring bar. 6N HCl solution is added dropwise until the pH is between 2 and 1 (foaming). 20 ml of ether is added to the beaker, and solid (powdered) NaCl is added until the aqueous phase is saturated. The mixture is transferred to a separatory funnel, and the aqueous phase is extracted 3 times with 50 ml of ether. The ether extracts are combined, dried over sodium sulfate, filtered, evaporated to constant weight, and the weight is recorded.

5. Recovery of the Basic Fraction. The pH of the acidic aqueous solution labeled "bases" is adjusted to between 12 and 13 by dropwise addition of 6 N NaOH under a layer of ether, saturated with sodium chloride, and extracted 3 times with 50 ml of ether; the ether extracts are dried over anhydrous sodium sulfate, filtered, reduced in volume, and weighed as above.

