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A ROUTINE WATER MONITORING TEST FOR MUTAGENIC COMPOUNDS

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ABSTRACT

We have developed a simple, relatively comprehensive method for the recovery of nonvolatile mutagenic compounds from surface waters. The method recovers compounds by sequential passage of the water sample through a silica gel bed (to mechanically filter the sample and to adsorb water-insoluble compounds such as polynuclear aromatic hydrocarbons), then a cation-exchange bed (to adsorb cationic and amphoteric compounds), and then an anion-exchange bed—all contained in a single multi-bed column of glass and teflon, the parfait column. Nonvolatile compounds not adsorbed to any of these beds (i.e., neutral, water-soluble compounds) were recovered following concentration of the column effluent by vacuum distillation at < 30°.

The beds of the parfait column were separated and eluted independently. Water-soluble ionic compounds were eluted with 2 M triethylammonium carbonate, and hydrophobic compounds were eluted with acetone. Under vacuum, the acetone or the components of the triethylammonium carbonate buffer (triethylamine and CO_2) were removed, leaving the nonvolatile components of the water sample in the residue. Acetone residues were taken up in dimethylsulfoxide; the others were taken up in water.

Using the Ames *Salmonella*/microsome reversion assay, each residue was assayed for mutagenic activity.

The method was evaluated by recovery of five known mutagens, benzo(a)pyrene, 4-nitroquinoline-1-oxide, ethidium bromide, nitrofurylfuramide, and sodium azide, each initially spiked into a sample of laboratory deionized water and an environmental water sample to a final concentration of less than 3 ppb. Recoveries were calculated from the mutagenic activity observed in the extracts, in comparison to the activity in parallel extracts of an unspiked water sample. Under these conditions, the parfait/distillation method was able to recover detectable mutagenic activity with three of the five mutagens tested.

The method has been used to survey ten Illinois surface waters for naturally occurring mutagenic activity. Samples from two sites, the Fox River at Aurora, Illinois, and the Salt Fork Creek at Urbana, Illinois, showed significant mutagenic activity.

The parfait/distillation method differs from other techniques for the recovery of waterborne mutagens in its emphasis on the recovery of nonvolatile compounds and neutral water-soluble compounds. This method has also detected significant mutagenic activity in samples as small as 2 gallons of water, a volume consumed by a normal person every few days.

This study represents the first step in the development of a routine method for the assay of mutagens in drinking water and drinking-water supplies. The results of this study and the strong correlation of mutagenic activity to carcinogenic potential raises the possibility that compounds present in surface waters may pose a chronic threat to the public's health.

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INTRODUCTION

The majority of human cancer is believed to be induced by chemical and physical agents present in the environment (Higginson and Muire, 1976). While research efforts continue to explore the nature of cancer in hopes of finding a cure, the presumed environmental etiology of cancer has suggested a means of prevention: reduce human exposure to carcinogenic substances in the environment. To implement this strategy requires the detection of carcinogenic substances in the environment so that their sources can be identified and the substances removed or exposure to them limited.

The identification of carcinogens in the environment can be achieved in either of two fundamental ways. First, environmental samples can be assayed for individual chemicals or classes of chemicals which have previously been found to be carcinogenic in animal cancer bioassays. This is the chemical approach. Second, the biological approach assays environmental samples for the ability to induce biological activities that are correlated with carcinogenic potential. Appropriate biological activities include damage to DNA, mutagenesis, and recombinogenic activity. The detection of these activities signals the presence of a potential carcinogen in the sample.

The chemical approach to the detection of environmental carcinogens is very powerful. Modern analytical chemistry provides sensitive methods for the extraction, separation and quantitative detection of specific chemicals from environmental matrices. Because the chemical being studied is known, each step in the procedure for recovery and analysis of the compound can be evaluated by reconstruction experiments. However, the chemical approach requires that the carcinogenic potential of each compound sought in the environment must have previously been evaluated in animal bioassay. Only approximately 6000 chemicals have ever been tested for carcinogenic activity, of which only 20 percent were found to be carcinogenic (National Cancer Institute, 1970-71). Since the total number of chemicals in common use in the U.S. approaches

63,000, a number that grows by about 1,000 per year (Maugh, 1978), the chemical approach cannot possibly be used for most environmental carcinogens—we simply do not have the resources to determine the carcinogenic potential of the 57,000 untested chemicals.

In contrast to the chemical approach, the biological approach is not tied to previous knowledge of a chemical's carcinogenic activity. A biological activity known to be correlated with carcinogenic potential is detected in the environmental sample, indicating the presence of one or more potential carcinogens in the sample. Since most chemicals have never been tested for carcinogenic activity, the biological approach should have a considerable advantage over the chemical approach for the detection of environmental carcinogens.

The comprehensive nature of the biological approach is also the source of its principal weakness. If carcinogenesis is not directly assayed, how predictive of carcinogenic potential is the biological activity that is assayed?

Among the three kinds of biological activity mentioned previously, only one—mutagenesis—has been extensively compared to carcinogenic potential. Assays of recombinogenic activity (in the form of the sister chromatid exchange assay in mammalian cells) are relatively new, although they show a strikingly high correlation with carcinogenic potential in the limited comparisons that have been made (Stetka and Wolff, 1976). Assay for DNA damage by the DNA repair test has been widely used in basic research on mutagenesis. DNA damage would probably correlate with carcinogenic potential about as well as mutagenesis, since DNA damage is considered to be a preliminary step in most pathways leading to mutation.

The comparison of mutagenic and carcinogenic potential carried out using the Ames *Salmonella*/microsome bacterial reversion assay has revealed an excellent qualitative correlation between mutagenesis and carcinogenesis (McCann *et al.*, 1975; McCann and Ames, 1977; Ames, 1977).

In a study of 175 carcinogens, Ames found that over 90 percent of them were detected in the mutagenesis assay. Conversely, less than 10 percent of 100 noncarcinogens tested were found to be mutagenic.*

Indeed, the strong correlation of mutagenesis with carcinogenesis has led to the formulation of a hypothesis that states that the first essential event in malignant transformation is a mutational event. This theory is known as the somatic mutation hypothesis. The theory predicts that mutagens are carcinogens.

Therefore, the use of an assay of mutagenesis in the biological approach to the detection of environmental carcinogens can be expected to work well. Any mutagenic activity detected can reasonably be expected to represent carcinogenic potential. In addition, the use of a test for mutagenic activity will probably detect far more carcinogenic chemicals than have as yet been identified as carcinogens by animal bioassay or epidemiological study. Finally, however, no single mutagenesis assay can be expected to detect all carcinogens—a small number of false negatives can be expected to occur.

Objectives

The work to be described in this report undertook to adapt a widely used test for mutagenicity, the Ames *Salmonella*/microsome reversion assay, for use with surface-water samples. The objective was to develop a test which could be applied to the routine monitoring of surface waters for mutagens.

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*Ames classifies as a mutagen any compound that acts directly to induce mutation or that is converted into an active mutagen through metabolism by an extract of liver enzymes added to the test plate. Properly speaking, compounds that *require* metabolism to be converted to the genetically active form are termed *pro*mutagens. From the perspective of mutagenic insult to humans, however, the distinction is essentially irrelevant since any promutagen entering the body would be acted upon by enzymes similar to those used in the Ames assay.

The specific objectives of the research included consideration of methods for the sterilization of the water samples (a prerequisite of the Ames assay), for the concentration of the organic materials present in the water (to provide sufficient mutagen to detect in the test), and for the evaluation of the quantitative limitations of the test. In this endeavor we proposed to use the information obtained from the USEPA study, "Monitoring to Detect Previously Unrecognized Pollutants"—the PUP project (Ewing *et al.*, 1977, p. 32). The PUP study attempted to identify and quantitate all of the organic constituents of surface-water samples collected from 200 sites nationwide. Our intention was to assay the PUP samples for mutagenicity with the Ames assay and to correlate the spectrum of chemicals previously identified in each PUP sample with the mutagenic activity observed. In this way we hoped to validate the detection of individual mutagens, to determine the quantitative limitations of the assay when applied to complex mixtures of organic chemicals, and to identify synergistic interactions and interferences among chemicals.

Literature Search for Citations of Mutagenic, Tumorigenic, and Teratogenic Activity of Compounds Identified in the PUP Study

Our study began with a search of the literature for citations of mutagenic activity in the Ames assay by the compounds identified in the PUP study. In addition we looked up those compounds in the National Institute for Occupational Safety and Health (NIOSH) *Registry of Toxic Substances* (1977), a compendium of the world's primary toxicological literature. Citations of tumorigenic, mutagenic, and teratogenic activity from the *Registry* were recorded in Table I. Of 143 compounds specifically identified in the PUP study, 33 (23 percent) were reported in the NIOSH *Registry* to exhibit at least one of these activities. Such a high percentage of compounds showing biological activity was frightening at first glance, but a more detailed evaluation of these citations allowed several distinctions to be made.

One third of the compounds in Table I were cited only for teratogenic activity. Teratogenic agents act during pregnancy to produce

Table I. Citations of Mutagenic, Tumorigenic, or Teratogenic Activity of Compounds Identified in the PUP Study

Chemical	IARC Designation	Ref.[a]	Mutagen	Tumorigen	Teratogen
Anthracene				c-rat[b]	
Atrazine		1	plant		
Benzene	suspected human carcinogen			c-hmn,t-mus	
2-benzothiazolethiol				t-mus	
Biphenyl				t-mus	
Bromodichloromethane		2	sal		
Caffeine					rat,mus,rbt
Carbon tetrachloride	positive animal carcinogen			t-rat,c-mus	rat
Chloroform	suspected animal carcinogen			t-rat,c-mus	rat
Chloroprene			rat		
1,2- dibromoethane		3	sal	c-rat,c-mus	
Dibutyl phthalate					rat
Dichloromethane		2	sal		
2,4-dichlorophenol				c-mus	
Dichlorophenoxyacetic acid					rat
Di(ethylhexyl) phthalate					rat
Diethyl phthalate					rat
Diisobutyl phthalate					rat
Dioctyl phthalate					rat
P-dioxane	positive animal carcinogen			c-rat,t-mus,t-gpg	
Ethylmethyl ketone					rat
Hexachlorobenzene					mus
Indole				t-mus	
Methylmethacrylate				c-rat	
Phenanthrene				t-mus	
Pyrene				t-mus	
Tetrachlorophenol (mixed isomers)				t-mus	
Tribromomethane		2	sal	c-mus	
Trichloroethylene	positive animal carcinogen	2	sal	c-mus	
2,4,6-trichlorophenol				c-mus	
2,4,5-trichlorophenoxyacetic acid				t-mus	rat,mus
Vinylidene chloride		2	sal		

[a]Reference to mutagenicity in the *Salmonella*/microsome assay: (1) Plewa and Gentile, 1976, (2) Simmon, Kauhanen, and Tardiff, 1977, and (3) McCann *et al.*, 1975. All other citations were from the National Institute of Occupational Safety and Health *Registry of Toxic Substances* (1977).

[b]mus = mouse, rbt = rabbit, sal = *Salmonella typhimurium*, gpg = guinea pig, hmn = human; t = tumor (whether malignant or benign is undefined), and c = cancer (a malignant tumor).

physical or functional defects in the fetus or offspring. Death *in utero* is a common measure of experimental teratogenesis. Because the development of the mammalian fetus during pregnancy is acutely sensitive to the surrounding chemical environment, many otherwise innocuous chemicals may exhibit teratogenic activity, particularly if administered under acute experimental laboratory regimes. Table salt (NaCl) and cane sugar (sucrose) are among the compounds that exhibit teratogenic activity under such conditions, and they are listed as experimental teratogens in the NIOSH registry. Teratogenesis is strongly dependent upon the dose of agent given, the route of administration and the stage of development of the fetus during administration. It was of concern therefore to note whether the experimental route of administration for the PUP compounds cited in the literature reflected likely routes of entry of environmental compounds and whether the experimental doses administered in studies cited were comparable to environmental concentrations found for the PUP compounds.

Among the teratogens in Table I, all of the phthalate esters cited were administered by intraperitoneal injection, a route normally unavailable to environmental compounds. The other teratogens listed were administered either in the diet or by inhalation. For those not injected, the dosages were between 1 percent and 30 percent of a lethal dose, apparently excessive in comparison to the levels found in surface waters. It is well established that there are threshold dosages for experimental teratogens; i.e., there are doses below which no teratogenic effects occur. The compounds found in the PUP study were present at concentrations substantially below those needed to induce experimental animal teratogenesis. Based on this knowledge, therefore, it seems unlikely that any of the compounds found in the PUP study pose a large teratogenic risk to the human population.*

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*We recognize, however, that this conclusion extrapolates experimental results from rodent to man and that there are substantial qualitative and quantitative differences in the teratogenic responses of primates compared to nonprimates. It should be noted that the majority of compounds found in the PUP study have never been tested for teratogenicity in primates, at any dose.

Considering next the significance of citations of tumorigenic and mutagenic activity, the problem of threshold concentrations becomes a serious concern. There is no universal agreement concerning the existence of thresholds for tumorigens or mutagens. At present the preponderance of scientific evidence and a strong concern for public health has led most toxicologists to favor the view that no thresholds exist. From this perspective the PUP compounds found able to induce tumors or mutations must be viewed as being potential toxins even in trace concentrations, their relative effectiveness being related in some quantitative way to their concentration.

Citations of mutagenic activity are also significant since there is strong evidence that the mutation of a cell is an essentially irreversible event; that is, mutations are accumulated over the lifetime of the cell. Mutations are also inherited by the descendants of the cell. Neglecting the possibility of thresholds, the yield of mutations induced in an organism is proportional to the concentration of the mutagen times exposure time. For the PUP compounds there could potentially be long exposure times to compensate for low concentrations, producing high levels of induced mutation. Finally, citations of mutagenic activity are significant to human health. The primary target of most mutagens is the DNA of the cell. Chemically all DNA is essentially the same, therefore an agent able to attack DNA leading to mutation in one organism has a high probability of being able to attack DNA and cause mutations in other organisms, including man.

These considerations, then, strongly suggest that the citation of a compound for tumorigenic or mutagenic activity in the NIOSH *Registry* should be cause for special concern about potential hazards that it may pose to human health. Yet there is another dimension to this information that requires consideration.

The bioassay of tumorigens requires careful experimental design to insure statistical validity, control of the purity of the chemical tested, control for spontaneous tumors, etc. Epstein has estimated

that only about half of all cancer bioassays so far conducted have met all of these criteria (National Cancer Institute, 1970-71; Epstein, 1977). Examples of noncomplying studies are to be found cited in Table I. For instance, 2,4,6-trichlorophenoxyacetic acid was cited in the *Registry* for tumorigenic and teratogenic activity. However, the *Registry* notes that the compound used in the study cited was contaminated with tetrachlorodibenzodioxins which by themselves could account for all the effects observed. Similar problems of purity may account for the citations of anthracene, phenanthrene, and pyrene as tumorigens in the NIOSH *Registry*; these compounds are generally not considered to be carcinogens, although they are closely related chemically to polynuclear aromatic hydrocarbons which are potent carcinogens. The International Agency for Research on Cancer (IARC) has undertaken the critical review of all available reports of cancer bioassays (World Health Organization, 1972-77) and has designated compounds for which sufficient critical data exist as positive, suspected, negative or indeterminate carcinogens. These designations are included in Table I.

From a conservative viewpoint, then, only five of the 143 PUP compounds deserve special attention as IARC proven or suspected carcinogens. To these might be added six others that have been found to exhibit mutagenic activity in the *Salmonella*/microsome assay. In summary, 11 of the 143 compounds identified in surface waters by the PUP study are known to be mutagens, suspected carcinogens, or confirmed carcinogens and deserve special attention as such. These compounds are potentially active at very low concentrations and may have a substantial deleterious influence on human health if in contact with people for long periods of time.

The Use of GC/MS Influences the Results of the PUP Study

A striking thing about all 11 of the most important PUP compounds (indeed about all the compounds listed in Table I) is that they are all relatively volatile; many are halogenated hydrocarbons. The PUP study utilized gas chromatographic mass spectrometry (GC/MS) as the means of

separation and identification of organics extracted from the water samples. This instrument best identifies volatile, organic-extractable compounds, so it is not surprising to find mostly volatile species on the PUP list of compounds identified. However, volatile organic-extractable compounds do not comprise all of the chemicals in surface waters. It was estimated by one analytical chemist involved with the PUP study* that no more than 30 percent of all compounds actually present in the water samples were detected. More recent estimates from similar EPA-sponsored studies puts the number of organics present that have been identified at about 10 percent. Amino acids are trivial examples of compounds normally present in surface waters at ppb concentrations that were not detected by the PUP study. These compounds are amphoteric and poorly volatile unless specifically derivatized.

Revised Research Plan

The PUP results obviously did not provide a sufficient catalogue of the compounds in the water samples to realistically predict the mutagenic response and/or synergisms and interferences in the Ames assay, as proposed in the original research plan. In addition, most of the compounds identified by the PUP study were volatile and thus would very likely have been wholly or partially lost during storage of the PUP water samples. Therefore we abandoned the use of the PUP samples and research results that was originally planned. The PUP data were retained only as an indicator of typical concentrations of trace organics expected in surface waters.

To meet the proposal's objectives, we decided first to undertake a study of the causes of systematic fluctuations in the Ames assay. It was anticipated that any routine monitoring procedure would provide only small amounts of organic materials from the water sample, leading to only small responses in the Ames assay. Under these conditions, the size of the systematic fluctuations in the assay would be the factor

*J. C. Means, 1977; personal communication.

determining the detection limit (sensitivity) of the test. Section II is a copy of a manuscript in preparation for *Mutation Research*, describing this work.

An additional revision of the research plan was made to meet the need for a comprehensive system for recovery and concentration of organic compounds from water. Several recovery methods have been used in other laboratories, including vacuum distillation, adsorption on macroreticular resins, ion exchange, reverse osmosis, solvent extractions, and adsorption onto activated charcoal. Each of these methods has particular advantages and drawbacks—none is ideal and comprehensive by itself. Vacuum distillation recovers all nonvolatile materials but concentrates all of the salts in water; the salts often interfere with subsequent bioassay or chemical analysis. The distillation process is slow; and the concentration occurs in the liquid phase so that hydrogen ions or trace metals and organic species are concentrated together several hundredfold, potentially accelerating chemical reactions among the compounds in the extract. Macroreticular resins are of great utility but are somewhat selective and require relatively long contact times (on the order of minutes) for effective adsorption of some materials. Ion exchange is obviously selective, but it is rapid and has high capacity. Reverse osmosis has a lower efficiency of recovery than the other methods mentioned here and requires very large volumes of sample, but it is otherwise relatively comprehensive and acceptable. Solvent extraction has been widely applied to water samples, as in the PUP project. It will not normally recover amphoteric and neutral, water-soluble compounds. The compounds that it does recover well are usually those most amenable to GC/MS and are therefore the ones currently best characterized. A similar statement can be made about highly volatile compounds that are recoverable by gas-stripping techniques. Charcoal adsorption shows some selectivity that is sometimes difficult to predict, and it is often difficult to recover the compounds from the charcoal.

From consideration of these methods, the PUP study, and the project's resources and overall objectives, the following goals were set:

1. Recovery of the least-well previously studied classes of compounds would be emphasized, meaning that recovery of volatiles would be ignored. The recovery of the neutral, water-soluble compounds would be most strongly emphasized, but amphoterics would also be emphasized.
2. In agreement with our objective of developing a test with the potential for routine use, the size of the sample was somewhat arbitrarily set at one to a few gallons (financial limitations also influenced this goal).
3. Recalling the typical concentrations of organics found in the PUP study, we set the detection of mutagen(s) initially present in the sample at a minimum of 1 ppb concentration as the goal for the detection limit of the method.

Rationale for the Design of a Comprehensive Recovery System

To design a comprehensive recovery system, each method mentioned above was reconsidered from the point of view of cooperative interaction with the other methods. Charcoal adsorption and solvent extraction were abandoned for the reasons stated. Vacuum distillation was considered of high potential for recovery of nonvolatile compounds and of greatest utility for recovery of neutral water-soluble compounds. To prevent interferences from salts, the material distilled could first be passed through beds of cation- and anion-exchange resins. Only removal of ionic material followed by reverse osmosis could be envisaged as able to recover neutral, water-soluble species as well. But this method would require sample volumes and ion-exchange bed capacities beyond the resources of this project. It was realized as well that macroreticular resins, solvent extraction, or charcoal could potentially be used in combination with cation and anion exchange plus vacuum distillation to remove special classes of materials from the ion-exchange effluent prior to vacuum distillation. But the minimal comprehensive train of steps seemed to be cation and anion exchange plus vacuum distillation. It was realized then, as well, that mechanical filtration would be required prior to ion exchange to trap sand, silt, and debris. For this purpose,

a silica gel bed was added to the proposed train; this bed would add the additional property of adsorbing highly water-insoluble materials such as polynuclear aromatic hydrocarbons before they could adsorb onto the polystyrene matrix of the ion exchangers.

Design of the Parfait Column

To implement the proposed train of recovery steps, we first attempted sequential passage of water samples through individual glass columns of silica gel, Amberlite IR 120 cation-exchange resin, and Amberlite IRA 400 anion-exchange resin. Flow rates through typical 1-cm-diameter cylindrical columns having 50-ml bed volumes were very slow. To process a one-gallon sample of deionized laboratory water required more than 8 hours, which was unsatisfactory for our purpose. To enhance flow rates and expose the sample to each adsorbent in sequence, several physical arrangements of the beds were considered. The stacking of beds in a single column was considered, but this arrangement posed the problem of recovering each bed independently of the others for subsequent elution. We considered columns that would open into two halves, exposing the stacked beds. We considered rectangular columns with 2 removable opposing sides at right angles to the direction of flow. We considered modular beds that could attach in a linear sequence, with large area for flow through the entry and exit orifices. Finally we considered beds stacked between spacers that were connected in turn to a central post that could be used to pull the entire assembly of beds out so that each bed could be recovered separately as it exited. Only the modular concept was as attractive as the stacked partitioned bed, or "parfait" column; but because of its simplicity the parfait column was the first to be attempted (Figs. 1A and 1B).

Consideration was given to the contact time necessary for effective adsorption of compounds onto each bed. Adsorption of polynuclear aromatics onto glass surfaces occurs rapidly (McGinnes and Snoeyink, 1974), although we have not found published reports of quantitative measurements of the minimum time required. Technical literature from

Rohm and Hass Corporation, the manufacturer of Amberlite analytical ion-exchange resins, indicates that exchange onto the immobile phase of Amberlite ion-exchange resins is (a) limited by diffusion rates and (b) limited by the accessibility of the solute to the ionic sites on the immobile phase. Constraint (b) depends strongly on the size and porosity of the ion-exchange bed and the column packing. With packings of IR 120 and IRA 400 prepared according to manufacturers' recommendations, effective exchange should be accomplished in one second or less. The spacers separating beds in the parfait columns were considered able to cause some channelization of the sample flow into the beds. Therefore, flow rates through the column were limited to a minimum of 30 sec contact with each bed. For the column dimensions initially chosen (4.8-cm-diameter cylinder with 50-ml minimum bed volume), this corresponded to a 100 ml/min maximum flow rate.

These flow conditions should strongly reduce the potential for channelization and allow sufficient time for complete ion exchange. The parfait column's design of individually recoverable, stacked beds can accommodate additional adsorbents such as charcoal and the macroreticular resins mentioned previously. In addition, it is possible to alter the column dimensions and bed volumes to increase the total volume of water sampled. It is only necessary when doing this to insure that the bed packing and flow characteristics in the bed permit effective contact with the adsorbent and that the time of contact of sample with the bed not limit adsorption. The use of macroreticular resins, which require long contact times relative to ion-exchange times, might necessitate much slower flow rates or much larger bed volumes to be effective.

Sequence and Capacity of Beds

The sequential multiple-bed concept must consider effects due to the order of the beds and must insure that no bed becomes saturated by any component of the sample.

The sequence of beds initially chosen (Fig. 1A) placed the silica bed first, to filter insoluble materials and to adsorb highly nonpolar compounds. No information on the quantitative capacity of silica gel to adsorb nonpolar compounds was available to guide us, so the bed volume initially chosen (50 ml) was arbitrary. Because the next two underlying beds contained a polystyrene matrix, it was recognized that nonpolar compounds passing through the silica bed might adsorb onto the ion-exchange beds by nonpolar rather than ionic interactions. To detect such compounds, an organic-elution step was added to the salt-elution steps for each of these beds to recover any such compounds (see *Sampling and Recovery*). In addition, it was considered possible that the capacity of the silica and the ion-exchange beds for nonpolar materials might be low enough (or the amount of nonpolar compounds high enough) that some material would enter the column effluent and be concentrated in the vacuum distillate. This would be apparent from the distribution of mutagenic activity in the organic eluate of all three column beds. However, no provision was made to recover poorly water-soluble material from the vacuum distillate.

The ion-exchange beds that followed the silica bed were placed with the cation-exchange bed (in the H^+ form) before the anion-exchange bed (in the OH^- form). This arrangement would prevent precipitation of metal hydroxides in the anion-exchange bed that might occur if this bed were put before the cation exchanger. We recognized that the indicated sequence would briefly expose anionic and neutral water-soluble compounds to low pH during passage from cation- to anion-exchange beds.

The sample volume was set arbitrarily at 2 gal (approximately 8 liters), and the ion-exchange capacity of the column was chosen to accommodate samples having a maximum of a 10 meq/l ion concentration. Before application to the parfait column, we estimated the conductivity of each sample with a Beckman model MG mho-gun portable conductivity meter, converting mho to the equivalent NaCl concentration at that temperature. The actual capacities of the ion-exchange beds were:

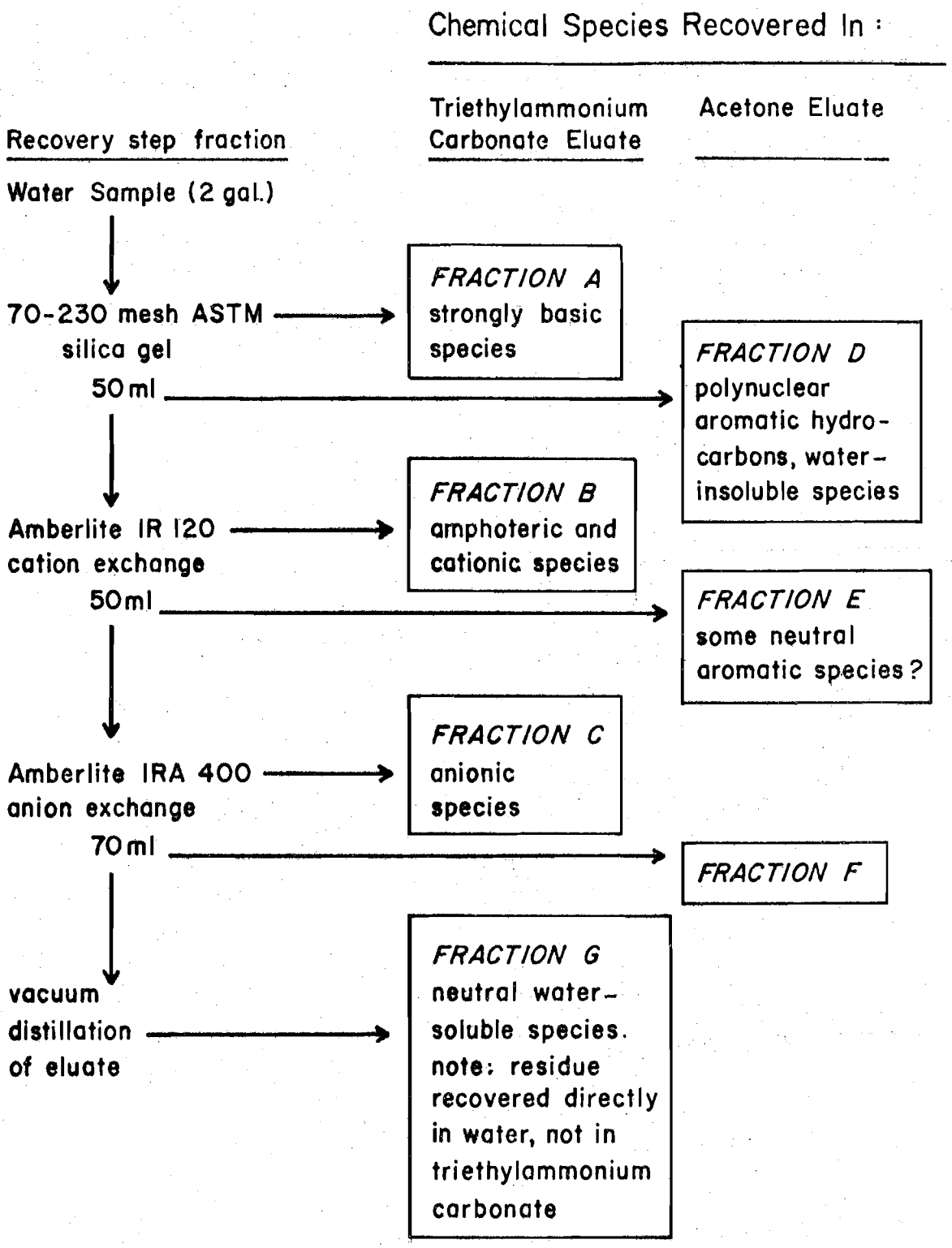

Figure 1A. Recovery of Mutagens by the Parfait Column Method

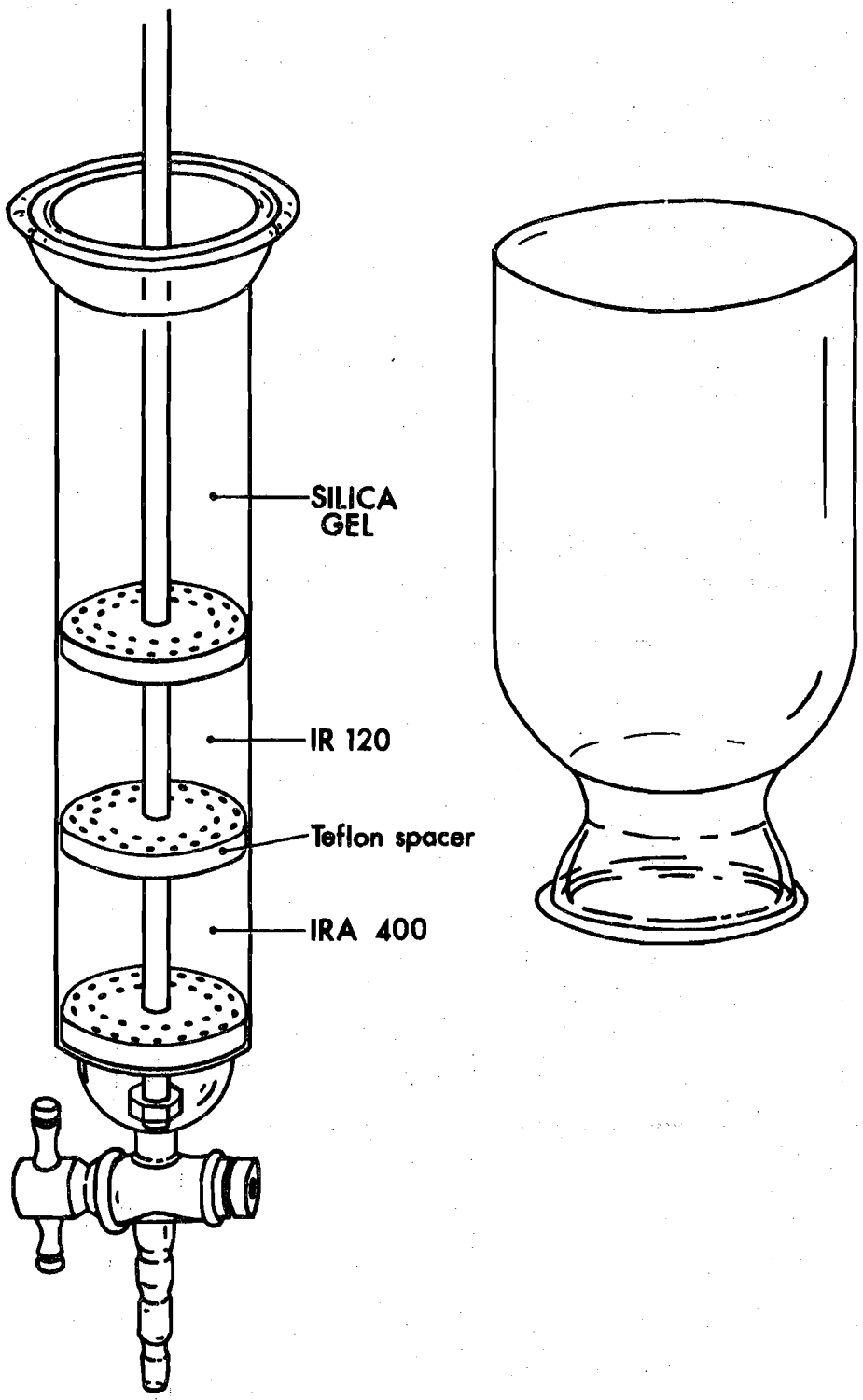

Figure 1B. The Parfait Column and Reservoir

IR 120: 1.75 meq/ml x 50 ml bed vol = 87.5 meq minimum
IRA 400: 1.2 meq/ml x 70 ml bed vol = 84 meq minimum

The volumes of samples with conductivities greater than 10 mM NaCl (equivalent) were reduced to accommodate the column ion capacity.

MATERIALS AND METHODS

Chemicals, Materials and Reagents

Benzo(a)pyrene [B(a)P] and ethidium bromide (EB) (2,7-diamino-10-ethyl-9-phenylphenanthridinium bromide) were obtained from the Sigma Chemical Co., 4-nitroquinoline-1-oxide (4-NQO) from ICN, and sodium azide (NaN_3, technical) from Eastman; 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2) was a gift from B. N. Ames. Silica gel 70-230 Mesh ASTM was obtained from EM Laboratories, Amberlite IR 120 and IRA 400 resins from Mallinckrodt. Dimethylsulfoxide (DMSO) was obtained from Mallinckrodt and sterilized in the autoclave before use.

Triethylamine (TEA) was distilled from the technical material; the fraction boiling at 88-90° was used. To prepare 2 M triethylammonium carbonate ($TEACO_3$), a water suspension of TEA (2 eq/l) was vigorously stirred with a magnetic stirring bar while CO_2 gas was bubbled through until pH 7.5 - 8.0 was reached. The CO_2 was generated from commercial dry ice in a stoppered flask and piped into the TEA suspension. Concentrated stock solutions of AF-2, 4-NQO and B(a)P were prepared by weighing crystals of the solid into a sterile tube and making up the solution with sterile DMSO. NaN_3 and EB were prepared similarly in sterile water.

Stocks of mutagens were stored in screw-capped tubes at 4° in the dark. Fiberglass prefilters (Nuclepore type 080, 4.7 cm diameter) were used to support the adsorption beds on the teflon spacers in the parfait column. All other chemicals used were commercial reagent grade.

Microbiological Methods

The rationale for the modifications of Ames' assay will be found in Section II. The methods used for the assay of mutagens from the parfait column are repeated here in detail.

Salmonella typhimurium, strains TA100, TA98, TA1535 and TA1538, were obtained from Professor B. N. Ames and tested for histidine requirement, ampicillin resistance, sensitivity to ultraviolet light, and deep rough character, as recommended by Ames (Ames, McCann, and Yamasaki, 1975). Frozen permanents were prepared from overnight L-broth (Lb) (Lennox, 1955) cultures and stored in 6 percent (^{v}/v) DMSO at -70°. Scrapings from the frozen permanent were transferred to 5 ml of medium containing Vogel Bonner E salts (Vb) (Vogel and Bonner, 1956) supplemented with 0.4 percent glucose, 0.3 mM L-histidine, 5 μM D-biotin, and 2 percent (^{v}/v) Lb (hereafter called enriched Vb) and cultured 16 to 20 h shaking at 37°. To prepare the inoculum for reversion assay, these cultures were harvested by centrifugation (5,000 x g for 5 min) and resuspended by vigorous vortex agitation in 2.2 ml 0.9 percent NaCl. The culture density was calculated from the A_{660} of a 1/25 dilution of this stock in a Bausch and Lomb Spectronic 20 colorimeter with an 18-mm colorimeter tube. In this instrument 1 x 10^9 bacteria/ml have an A_{660} of about 1.5. The stock was diluted to a calculated density of 1 x 10^9 bacteria/ml and put on ice; 0.10 ml of this suspension was applied to each assay plate. Top agars were prepared as described by Ames except that the final 2 ml of top agar contained a total of 50 nmoles L-histidine for TA100 and 100 nmoles L-histidine for all other strains. Vb basal agars (VbA), the S-9, and the S-9 mix were prepared exactly as recommended (Ames, McCann, and Yamasaki, 1975).

Pipetman or Eppendorf automatic pipets with sterile tips or glass pipets coupled to propipetter bulbs were used to dispense all mutagens and environmental samples—no mouth pipetting of these materials was permitted. Additions to the 46° molten top agar were made in order of increasing heat lability: mutagen or sample, then inoculum, then S-9 mix. Top agars were quickly mixed on a vortex mixer and plated on

VbA. Revertant colonies were scored after 72 h incubation at 37°. Controls for the induced and spontaneous mutation frequency of each strain, for sterility of samples and stock solutions, and for the activity of the S-9 mix were routinely included in every experiment. The stock inoculum was titered at the end of each experiment by plating appropriate dilutions onto L-agar and onto VbA supplemented with 0.3 mM L-histidine and 5 mM D-biotin.

Bioassay for histidine in environmental samples was carried out as described in Section II, except that TA1538 was usually substituted for TA100. Typically 0.50 and 0.050 ml of unknown were assayed simultaneously.

Preparation of the Parfait Column

All glassware was cleaned with chromic-acid solution. Silicate accumulations remaining after chromic-acid cleaning were removed by bathing the accumulation in 7 N NaOH for several hours, rinsing, neutralizing with 6 N HCL, and rinsing in water. All glassware was finally rinsed thoroughly with tap water followed by three or more rinses with water purified with Barnstead D8901 high capacity and D0809 ultrapure ion-exchange cartridges. This water, hereafter called DI water, had a resistance in excess of 0.5 megohm.

Ion-exchange resins for the parfait column were washed before use with 10 changes of reagent grade methanol and stored at room temperature in this solvent. Silica gel was dusted into deionized water just prior to pouring the parfait column.

To prepare a column, the bottom teflon spacer was threaded onto the central rod, followed by a Nuclepore fiberglass prefilter, to act as a support for the ion-exchange resin. The assembly was seated in the bottom of the column, which was about half full with deionized water. The 70-ml IRA 400 bed was then washed into the column with a stream of DI water from a wash bottle and rinsed down the sides of the column into place. Another prefilter was threaded down the rod onto the bed, followed in

turn by a teflon spacer and another prefilter. In a similar fashion the 50-ml IR 120 bed was added, then the 50-ml silica bed. The silica bed was topped with a prefilter only. Two bed volumes of DI water were then passed through the column before application of the sample.

Sampling and Recovery of Extracts for Mutagenicity Assay

Water samples were collected in glass gallon bottles. Old bottles were cleaned with chromic acid before reuse; new bottles were used without cleaning. It had been determined in the PUP project that new bottles were free of contaminating organic materials (Ewing *et al.*, 1977, p.32). The bottles were uncapped at the sampling site and filled to the neck with the sample, then recapped and returned to the lab. If transportation required more than 2 hours, the bottles were buried in crushed ice in a styrofoam cooler for transportation.

In the lab the reservoir was attached to the parfait column, and samples were poured into the column to near the brim of the reservoir. Samples to be spiked with mutagen were opened in the lab; the mutagen was added and mixed with a teflon-coated magnetic stirring bar just prior to application to the column. Conductivity of each sample was determined with the Beckman mho-gun newly standardized with 5 and 10 mM solutions of NaCl. The effect of temperature on the conductivity of NaCL standard was not found to be significant between 0-23° and between 0-10 mM NaCL; therefore corrections for differences in the temperature of standard and sample were not made. If any sample showed a conductivity equivalent to 8 mM NaCL or greater, its volume was reduced from the nominal 2-gal sample size to avoid titration of the ion-exchange beds.

Samples that had appreciable sediment or suspended solids often clogged the top of the silica bed, reducing the rate of flow through the column. When this occurred, a long 1-cm-diameter teflon rod was used to dislodge the sediment and redistribute it into a ring around the circumference of the top of the silica bed. Even so, some samples required as much as seven hours to pass through the column.

The effluent from the column was collected in gallon bottles that were cleaned in the same manner as those used to collect samples. The effluent was then transferred to two 2-liter round-bottom flasks (filled about 3/4 full), and these flasks were attached to a Labconco Freeze Dry 5 lyophilizer by a glass adaptor through a 24/40 standard taper joint sealed with a tapered teflon sleeve. No vacuum grease was used. The sample was degassed and then cooled by vacuum distillation. When bumping had ceased, the flasks were immersed in a 30° water bath which heated the sample to permit distillation and prevented the formation of ice in the flask. Using these methods in addition to cleaning the ice out of the lyophilizer cold trap every 12 hours and changing pump oil weekly, it is possible to distill 1 gallon of water to dryness every 24 hours. Samples awaiting distillation were stored in the dark at 2° in the capped gallon bottles.

Complete parfait columns were usually stored overnight at 2° before elution; some columns were stored for three days at 2°.

Recovery of the column beds was accomplished by pulling the central rod until each bed was exposed in turn at the top of the column. It was necessary to open the stopcock at the bottom of the column to admit air before pulling out the beds. Occasionally the bed assembly would not move easily when pulled. It was found that 2 to 5 min chilling of the whole column in a -20° freezer always loosened the assembly and eased bed sliding. As each bed appeared near the top of the column, it was pulled about halfway out, leaving the spacer at the bottom of the bed firmly in contact with the column wall. Using a stream of DI water from a wash bottle, the top bed was sluiced into a Buchner funnel with a coarse ground-glass frit that stood above a 500-ml round-bottom flask. The silica bed was recovered with its fiberglass prefilters; the prefilters from the ion-exchange beds were not included in the material to be eluted.

Having been recovered separately, each bed was first eluted with four 50-ml volumes of 2 M $TEACO_3$ buffer. To elute, the buffer was poured

through the ion-exchange or silica adsorbent in the Buchner funnel, and this eluate was combined with the original water washings from the transfer of the bed to the funnel. When the material in the Buchner funnel had run dry by gravity, the round-bottom flask containing the eluate was removed and stored at -20° until it could be lyophilized/ vacuum distilled to dryness.

A new, clean 250-ml round-bottom flask was then put into position, and the adsorbent was eluted with four 50-ml volumes of reagent acetone. In addition, acetone washings of the empty parfait column, the reservoir, and the bottles that contained the original water sample were added to the silica acetone eluate. Highly water-insoluble compounds such as the polynuclear aromatic hydrocarbons may adsorb on glass surfaces, so acetone washings were used to recover these materials that were adsorbed from the sample before it reached the silica bed.

The acetone eluates were first reduced in volume in a rotary evaporator with a 30° water bath and ice trap and then taken to dryness on the Labconco freeze-dryer. This residue was transferred by several acetone washings to a 20 x 125-mm screw-cap tube. The tube was attached to the lyophilizer, and the vacuum was very gently bled open to distill off the acetone. When dry, the residue was dissolved in 0.80 ml sterile DMSO and stored at -20° until assay.

The $TEACO_3$ eluates were distilled to dryness on the lyophilizer after the aqueous column effluent had been completed. In this way all of the $TEACO_3$ buffer was removed. These residues were taken up in several water washings of 4 ml each and transferred to large test tubes in a final volume of no more than 16 ml. These solutions were then centrifuged at 5,000 x g for 5 min if they contained insoluble material; and the supernatant was sterilized by driving it through Nuclepore 0.2-mm filters with a hand-held plastic syringe. The weight of the sterile filtrate was determined by difference and then made up to 8, 12 or 16 g by addition of sterile water. The extracts were stored in sterile 20 x 125-mm screw-cap tubes at -20° until assay.

Data, Standards, and Significance

To determine the recovery of a spiked mutagen, a standard dose-response curve of that mutagen was included in the experiment. Positive controls in all other experiments were 2 μg B(a)P with S-9 mix (TA98) and 10 ng 4-NQO without S-9 mix (TA100).

According to Ames, a significant result is one in which the reversion frequency on an experimental plate is greater than two times the frequency on the appropriate control.

In our work, the variation in spontaneous background frequency was reduced when histidine addition was closely controlled and a standard inoculum was used (Section II). Nevertheless, we also used Ames' criterion (above) for the minimum significant response.

When scoring plates which showed contaminating bacteria, the following system was used. No special designation was made if only a very few contaminating colonies were present, if their morphology was easily distinguished from the *Salmonella* morphology, and if the colonies were well separated from the *Salmonella*. If the contaminants overlapped any *Salmonella* colonies or were numerous, the score was reported as revertants "plus C." This designation was intended to mean that although the scorer felt that all of the *Salmonella* were scored, it was possible that the contaminant obscured or interfered with the count of *Salmonella*; and therefore the result must be interpreted with caution. Finally, if the contaminant clearly prevented the accurate counting of the *Salmonella*, the report stated "Contam."—or if confluent, "Conf. contam."

RESULTS

Three sets of experiments were performed to establish the feasibility of recovery of mutagens by the parfait column. First,

recovery of mutagens added to laboratory deionized water was attempted to demonstrate the method's limitations under ideal conditions. Recovery of mutagen was then attempted from an environmental surface water with two parfait columns in parallel: one spiked with mutagen and one as control for mutagens occurring naturally in the water. Finally, a variety of essential control experiments was performed specifically to determine if histidine were contributing to the responses observed and whether ionic strength or chromate carry-over from the cleaning solution could have influenced the result.

In the following tables, the average revertants per plate are reported for each of the seven fractions obtained from the parfait column (Fig. 1A). Details of the sample origin and amount of mutagen spiked are reported at the top of each table. Each table is followed by the standard curve for the mutagen spiked into that sample. Table 7 shows percent recovery of the mutagen from each fraction, based on the data in the preceding tables and standard curves.

In all tables, Fractions A and H are the $TEACO_3$ eluate of the silica bed, B and I the $TEACO_3$ eluate of the IR 120 bed, C and J the $TEACO_3$ eluate of the IRA 400 bed, D and K the acetone eluate of the silica bed, E and L the acetone eluate of the IR 120 bed, F and M the acetone eluate of the IRA 400 bed, and G and N the vacuum distillate of the parfait-column eluate. For each fraction, 1/8, 1/80, and sometimes 1/800 of the total volume of the fraction was assayed in duplicate. The average of the duplicate assays is reported. Also, single plates without *S. typhimurium* but carrying 1/80 vol of each fraction were plated as sterility controls; sterility results are reported only if not zero. Spontaneous reversion frequencies for each strain are given in the column headed 0 volume; positive controls for each strain and for S-9 mix activity are reported at the bottom of each table.

Elution of Control (No Mutagen) Column

The first table describes an experiment to determine whether components of the deionized water, parfait column beds, elution buffers, glassware, etc. could produce either mutagenic or toxic responses in the Ames assay. A white, acid-stable, water-soluble material was found in the IRA 400 $TEACO_3$ eluate (C), and a smaller amount was found in the column-effluent fraction (G). This material made Fraction C so viscous that filter sterilization was not possible. No mutagenic activity was detected in these or any other fraction from the column, although the material in Fraction C depressed the spontaneous reversion frequency of TA100 (Table 1). The material in Fraction C was decomposed by hydrofluoric acid and was therefore tentatively identified as silicic acid. This acid could have eluted from the silica gel bed and adsorbed by ion exchange onto the anion-exchange bed. In all parfait columns used to date, this material has been present in the IRA 400 $TEACO_3$ eluate. Preliminary experiments with this material demonstrated slight depressions of spontaneous and induced reversion frequencies (data not shown). In environmental samples (Tables 8-12) the material probably contributed to the frequent finding of toxicity in Fraction C, perhaps by synergistic action of the silicate with natural anionic toxicants. Even though unsterilized, the C fractions in these experiments usually did not show unacceptable numbers of contaminants in the sterility controls.

Recovery of Mutagens Spiked into Deionized Water

The mutagens tested for recovery from deionized water were B(a)P, EB, and NaN_3 at 2.6 ppb (20 µg/2 gal) and AF-2 and 4-NQO at 1.3 ppb (10 µg/2 gal). From consideration of structure and solubility, B(a)P was expected to adsorb onto the silica bed and elute into acetone, EB to adsorb onto IR 120 and elute into $TEACO_3$, 4-NQO and AF-2 to be found in the column effluent, and NaN_3 to adsorb onto the IRA 400 bed and elute into $TEACO_3$.

Table 1. Mutagenicity of Control Parfait Column
No mutagen added to 2 gal deionized water

Revertants of:		TA98 with S-9 mix			TA100 with S-9 mix		
Fraction	Vol extract:	0	1/8	1/80	0	1/8	1/80
A		42	52	33	120	106	101
B			33	34		133	105
C			34	53		57	106
D			33	38		126	104
E			30	36		90	111
F			45	45		106	124
G			39	37		90	114

Positive controls: TA98 with S-9 mix and 2.0 μg B(a)P=368
TA100 with 10 ng 4-NQO, without S-9 mix=420

B(a)P partitioned as expected, and 68% of the applied mutagenic activity was recovered (Tables 2 and 7). Note that one composite standard curve (Fig. 2) was used to calculate recoveries for all B(a)P experiments (Tables 2 and 8).

EB was found mainly in the $TEACO_3$ eluate of the silica bed and to a smaller extent in the $TEACO_3$ eluate of the IR 120 bed (Table 3). Considering the structure of EB, this behavior can be rationalized even though the compound did not behave as expected. The adsorption of EB onto silica probably occurred as a result of weak ionic and hydrophobic interactions of the EB with the silica. It should be emphasized that even though the EB did not adsorb where anticipated, it did partition strongly into one of the 7 fractions of the parfait column. It is absolutely necessary that compounds partition strongly for the method to work. The aqueous fraction (G) from the EB trial was not collected, so any unadsorbed material that reached that fraction would not have been detected.

Table 2. Recovery of Benzo(a)pyrene from Deionized Water

20 µg B(a)P added to 2 gal water

	Revertants of TA98 with S-9 mix		
Fraction	Vol extract: 0	1/8	1/40
A	38	40	38
B		42	40
C		47	28
D		246	59
E		49	32
F		44	48
G		ND	ND

Positive control: see standard curve, Figure 2

Sterility control: 1/40 vol of B=1, C=1, D=1

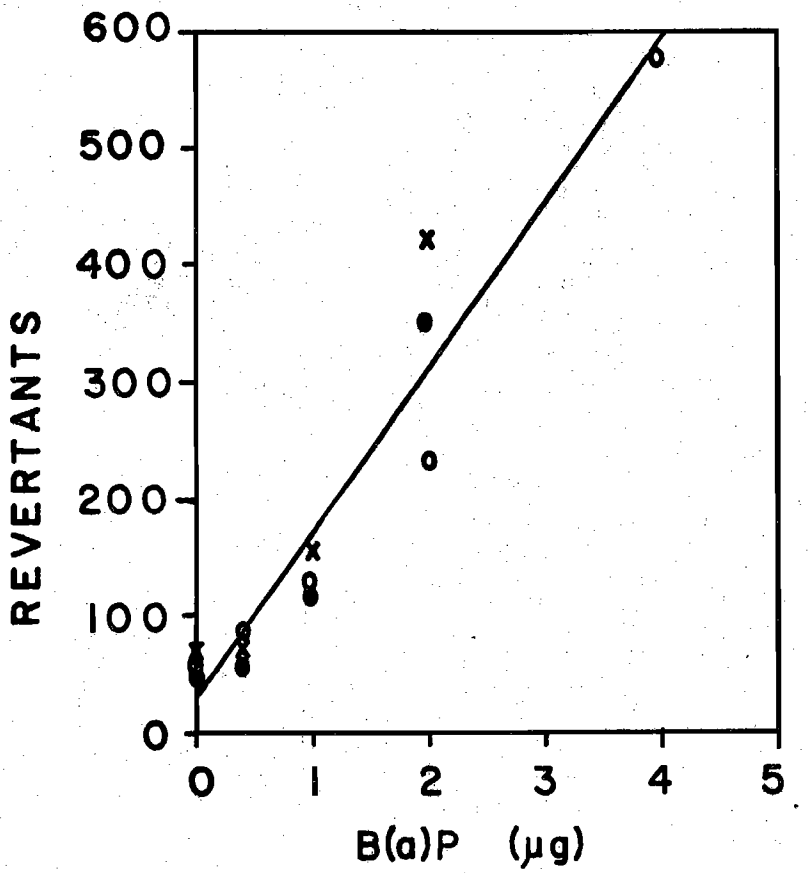

Figure 2. Response of TA98 to B(a)P and S-9 Mix

Table 3. Recovery of Ethidium Bromide from Deionized Water

20 µg EB added to 2 gal water

	Revertants of TA98 with S-9 mix		
Fraction	Vol extract: 0	1/8	1/40
A	41	223	87
B		86	52
C		56	58
D		62	58
E		48	51
F		49	46
G		ND	ND

Positive control: see Figure 3

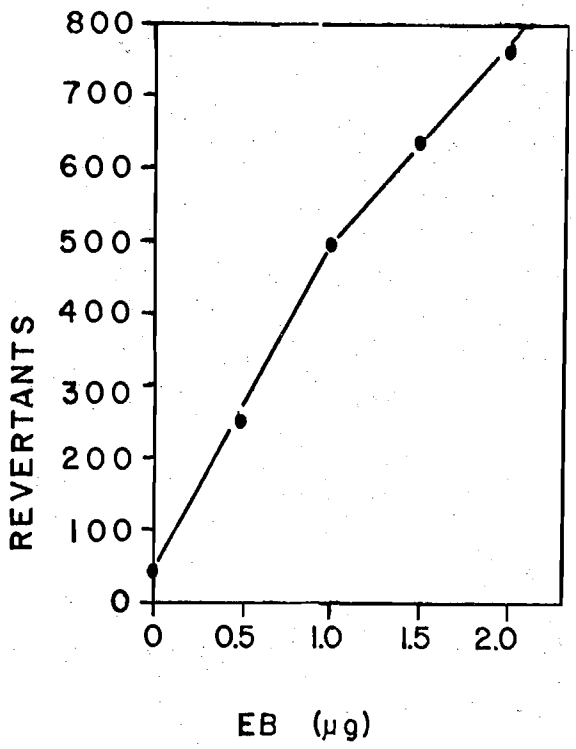

Figure 3. Response of TA98 to EB and S-9 Mix

4-NQO and AF-2 partitioned as expected into the aqueous fraction, with minor amounts found respectively in the IR 120 $TEACO_3$ and silica $TEACO_3$ fractions (Tables 4 and 5).

As shown in Table 6, NaN_3 was not detectably recovered from the parfait column; the data in this table also exhibit uncharacteristically large variations. Consider the apparently significant response of Fraction C, 1/80 vol: from the standard curve one is tempted to conclude that this assay plate contained the minimum detectable amount of NaN_3. The 1/8-vol assay could then be interpreted to contain a toxic amount of NaN_3. The standard curve shown in Figure 6 does not show whether the reversion frequency induced by azide becomes depressed in the range 100-300 ng NaN_3 on the plate. Independently constructed standard curves indicate that it does not; i.e., the frequency continues to rise in this range, although not linearly. Therefore we conclude that the value for 1/80 vol of Fraction C actually represents an extreme fluctuation rather than recovered azide.

Considering the likely path of NaN_3 through the column, it would first be expected that the Na^+ ion would exchange with a proton on either the silica or IR 120 bed. It is possible that the resulting decrease in pH might have activated the azide ion, permitting the reaction of HN_3 with the column bed or other organic materials in the sample. Therefore the azide may have given rise to nonmutagenic, nonionic species unable to exchange with OH^-. An alternative explanation might be that during sample work-up the volatility of HN_3, found even in small amounts in the $TEACO_3$ eluate, would result in the distillation of the azide out of the sample. To examine the possibilities in the future, it would be interesting to substitute the $TEAH^+$ form of the cation-exchange bed for the H^+ form to prevent the transient drop in pH that occurs during passage of compounds from cation- to anion-exchange beds.

Table 4. Recovery of 4-nitroquinoline-1-oxide from Deionized Water

10 µg 4-NQO added to 2 gal water

Revertants of TA100 without S-9 mix				
Fraction	Vol extract: 0	1/8	1/80	1/800
A	232	226	232	199
B		426	229	238
C		163	233	230
D		207	238	240
E		206	223	218
F		206	222	211
G		3124	1736	310

Positive control: see Figure 4

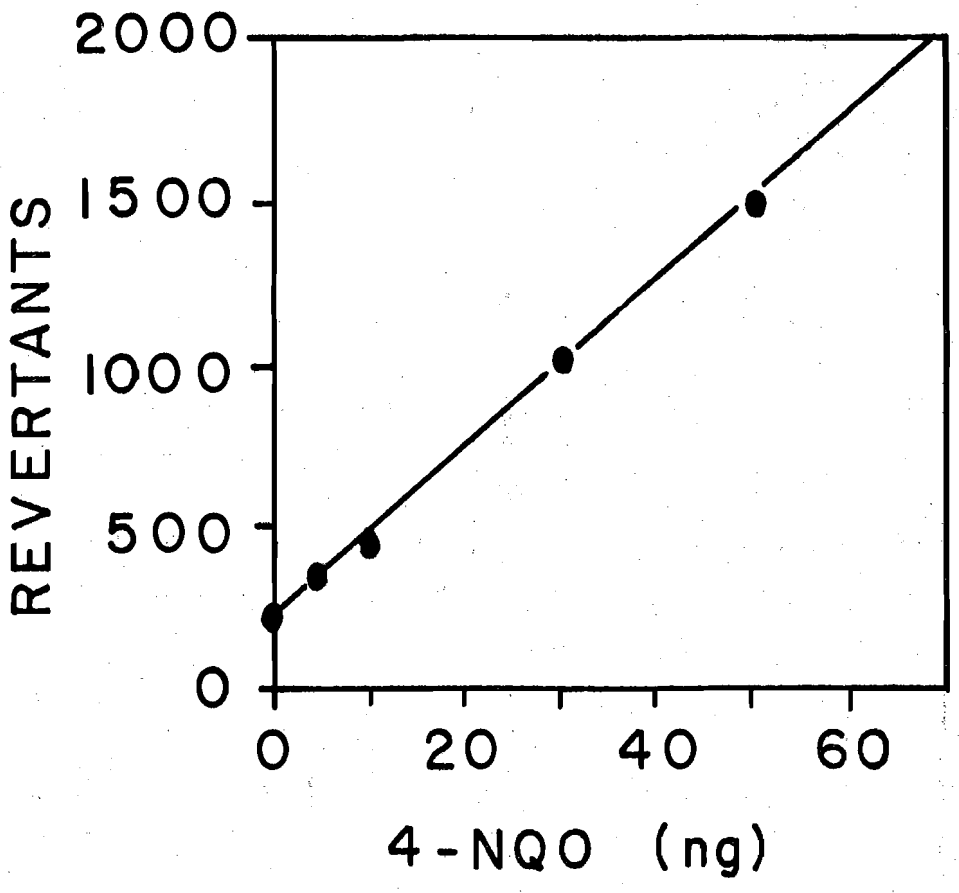

Figure 4. Response of TA100 to 4-NQO

Table 5. Recovery of 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide from Deionized Water

10 µg AF-2 added to 2 gal water

Revertants of TA100 without S-9 mix				
Fraction	Vol extract: 0	1/8	1/80	1/800
A	117	609	149	122
B		168	131	106
C		131	116	118
D		120	115	106
E		128	122	132
F		168	115	112
G		1324	240	126

Positive control: see Figure 5

Sterility control: 1/80 vol of D=1, G=1

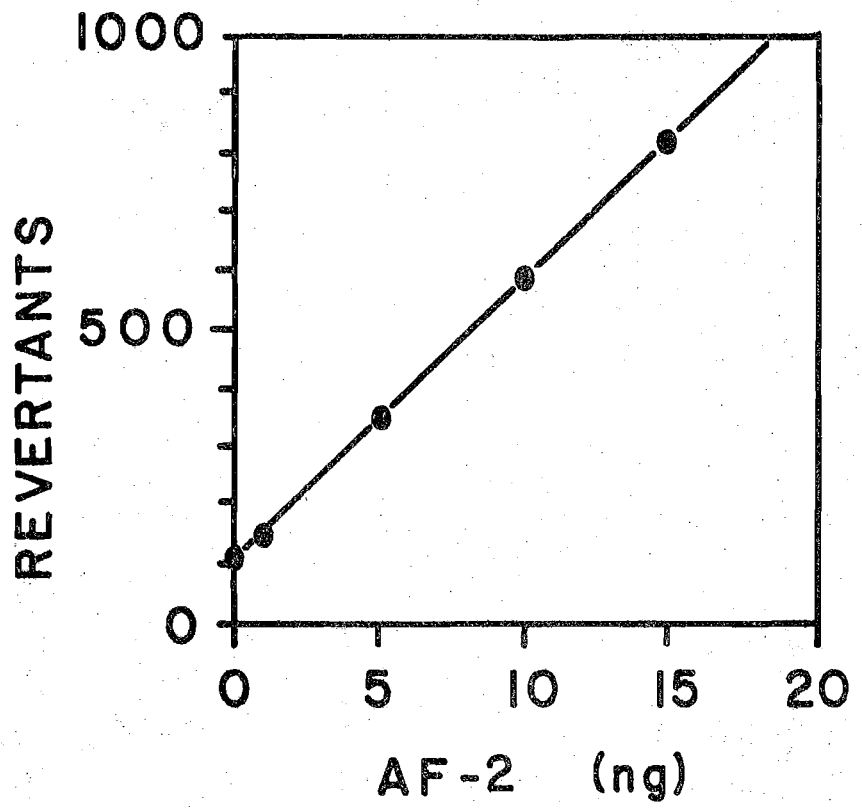

Figure 5. Response of TA100 to AF-2

Table 6. Recovery of Sodium Azide from Deionized Water

20 μg NaN_3 added to 2 gal water

	Revertants of TA1535 without S-9 mix			
Fraction	Vol extract: 0	1/8	1/80	1/800
A	119	214	145	168
B		28	157	226
C		55	244	184
D		170	240	157
E		195	167	168
F		136	196	166
G		127	177	204

Positive control: see Figure 6

Toxic fractions: B, C

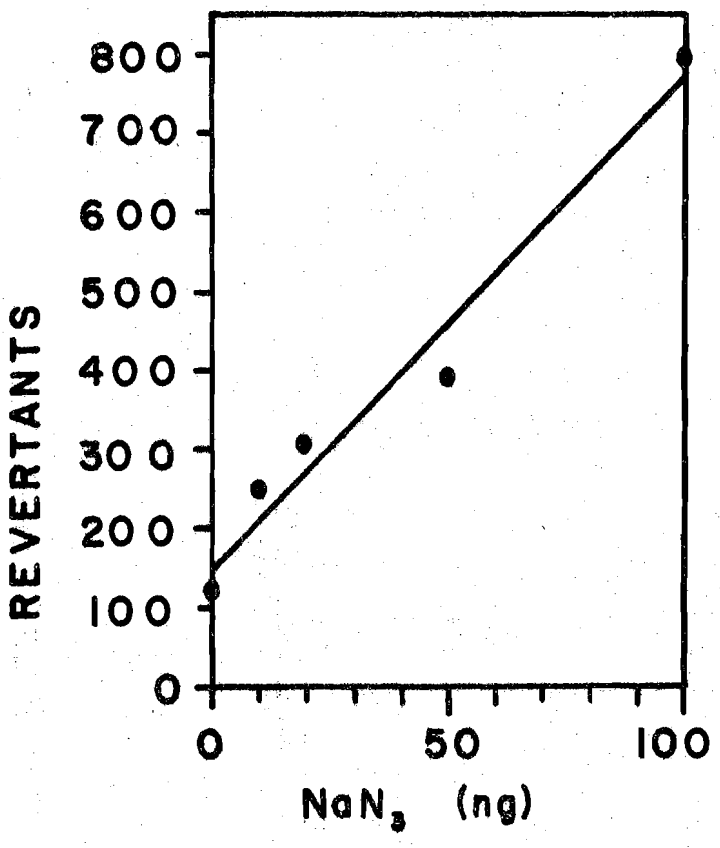

Figure 6. Response of TA1535 to NaN_3

In summary, 4 of the 5 mutagens tested with deionized water were recovered and detected by mutagenicity assay. The recoveries ranged from 62% for B(a)P to 3.2% for AF-2 (Table 7). The fifth mutagen, NaN_3, was not detectably recovered. Retrospective consideration of the recovery conditions suggested that the azide was lost either by reaction with components of the column at acidic pH or by volatilization during work-up.

Recovery of Mutagens from Environmental Waters

Having established that some of the mutagens tested could be recovered from purified water, recovery of the mutagens was attempted from environmental water. To control for naturally occurring mutagens in the water, two columns were run in parallel—one spiked with mutagen and the other not (Tables 8-12). Interesting variations in the recoveries of all the mutagens occurred, compared to the recoveries from deionized water.

The recovery of B(a)P (Table 8) closely resembled the result from deionized water (Table 2). Note, however, that the spike of B(a)P apparently produced a toxic product in Fraction B (Table 8) compared to Fraction I. It is possible that during sampling and/or work-up, some of the B(a)P was chemically or biologically altered to a toxic form which bound to the cation-exchange bed. The possibility of alteration of spiked mutagen during sampling/work-up will be mentioned again for all of the other mutagens tested.

It does not seem unreasonable to expect that these biologically active molecules could be transformed by enzymes from microbes or other organisms (insect larvae etc.) in the water samples during the 4-6 hours required to apply the sample to the parfait column. Future work with radiolabelled mutagens will examine this possibility. For now, note that a significant amount (72%) of the mutagenic activity applied as B(a)P was recovered. The activity was found in Fraction D, the same fraction in which it was found when recovered from deionized water (Table 2).

Table 7. Summary: Percent Recovery of Mutagens from Deionized Water

	% Recovery of Added Mutagen				
Fraction	B(a)P	EB	4-NQO	AF-2	NaN_3
A	<10 [a]	17	< 0.6	0.9	< 2
B	<10	5	0.6	< 0.2	< 2
C	< 10	< 4	< 0.6	< 0.2	< 2
D	68	< 4	< 0.6	< 0.2	< 2
E	< 10	< 4	< 0.6	< 0.2	< 2
F	< 10	< 4	< 0.6	< 0.2	< 2
G	< 10	ND	51.2	2.3	< 2
Total	68	22	52	3.2	Not detected

[a] The number following the < symbol is the detection limit of the assay, expressed as a percentage of the mutagen added to the water sample.

The recovery of EB from Boneyard Creek Water (Table 9) showed several interesting features. First, the control column extract, assayed with TA100, showed unusually high background (control) reversion frequencies that essentially obscured the results of the TA100 assay. Second, the control extract assayed with TA98 (Fractions H-N) showed one fraction, N, which exhibited significant spontaneously occurring mutagenic activity. Next, the ethidium bromide recovered from the spiked sample (Fractions A-G) was found predominantly in the cation-exchange acetone eluate, Fraction E, and the neutral, water-soluble fraction, G. Recovery in these fractions differs from recovery from deionized water (Table 3). Finally, the total mutagenic activity recovered was over eight times that applied to the column.

Table 8. Recovery of Benzo(a)pyrene from Boneyard Creek Water

20 µg B(a)P added to 2 gal water (Fractions A-G)

Revertants of:		TA98 with S-9 mix			TA100 with S-9 mix		
Fraction	Vol extract:	0	1/8	1/80	0	1/8	1/80
A		62	2	75+C		ND	ND
B			0	65		ND	ND
C			81+C	62+C		ND	ND
D			258	58		ND	ND
E			67	50		ND	ND
F			60	62		ND	ND
G			29	60		ND	ND
H			52	60	133	130	156
I			62	66		143	133
J			110+C	55+C		133+C	112+C
K			72+C	61		92+C	144
L			50	54		130	131
M			55	58		153	160
N			41	58		142	127

Positive controls: TA98, see Figure 2
TA100 with 20 ng AF-2, without S-9 mix=1075

Sterility controls: 1/80 vol of A=2, C=4, J=1, K=2, N=1

Very high levels of spontaneous reversion of TA100 (and other strains) have very occasionally been observed in our laboratory. Usually, it has been possible to trace the cause to a procedural error in the experiment or to the presence of a contaminating strain morphologically indistinguishable from *S. typhimurium*. The result recorded in Table 9 was included to illustrate the critical value of the sterility and spontaneous reversion controls.

EB was recovered from the environmental water sample in the acetone eluates of the silica (Fraction D) and the IR 120 (Fraction E) resin. When recovered from deionized water, the EB was found in the

Table 9. Recovery of Ethidium Bromide from Salt Fork Creek Water

20 µg EB added to 2 gal water (Fractions A-G)

Revertants of:		TA98 with S-9 mix			TA100 with S-9 mix		
Fraction	Vol extract:	0	1/8	1/80	0	1/8	1/80
A		48	81+C	60+C			
B			3+C	74			
C			0+C	64			
D			316	71			
E			700	460			
F			114	86			
G			569	629			
H			77	50	639	88+C	770
I			26	42		0+C	662
J			2	48		ND	525
K			71	65		522	648
L			48	66		610	689
M			64	66		631	620
N			187	53		375+C	632

Positive controls: TA98, see Figure 7
TA100 with 20 ng AF-2, without S-9 mix=750

Sterility controls: 1/80 vol of C=1

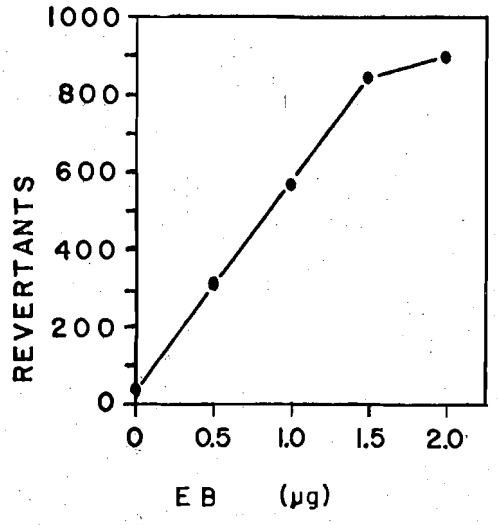

Figure 7. Response of TA98 to EB and S-9 Mix

$TEACO_3$ eluates of the silica (Fraction A) and the IR 120 (Fraction B) resin. It seems likely that increasing competition for ionic sites on the silica and IR 120 resins was responsible for this shift. Also, it is not clear whether the EB remained in the ionized form in the environmental water sample; a large fraction of the mutagenicity in this experiment was recovered in the neutral water-soluble fraction. Clearly, these observations require further study before they can be understood.

More intriguing is the tremendous increase in total mutagenic activity recovered. Two possible explanations are immediately apparent. Ethidium bromide by itself is inactive as a mutagen but gives rise to mutagenic activity if activated by a microsomal enzyme preparation (MacGregor and Johnson, 1977). Biological or chemical transformation of the compound during sampling and work-up could possibly have produced a derivative of EB that was intrinsically more mutagenic than the microsomal products, that was the precursor of such a derivative, or that increased the final amount of microsomal mutagenic product. Alternatively, an error in spiking could have put much more EB into the sample than planned. Both explanations can be tested in the future by repeating the experiment.

The attempt to recover 4-NQO from Salt Fork Creek water failed to detect mutagenicity. However, toxic activity was found in Fraction G, where the 4-NQO was expected. 4-NQO is known to be rapidly taken up by *S. typhimurium**, and it may therefore be expected to interact as well with the microbes present in the environmental water. Tentatively, we conclude that the result in Table 10 represents metabolism of 4-NQO to a mildly toxic, nonmutagenic derivative.

In the first trial recovery of AF-2, one each of the two spiked gallon bottles of water were mistakenly applied to each column (Table 11). Thus 5 µg of compound was applied to each column rather than 10 µg to one

*B.W. Penman, 1979; personal communication.

Table 10. Recovery of 4-nitroquinoline-1-oxide from Salt Fork Creek Water

10 µg 4-NQO added to 2 gal water (Fractions A-G)

	Revertants of TA100 without S-9 mix		
Fraction	Vol extract: 0	1/8	1/80
A	92+C	120+C	124+C
B		118+C	130+C
C		0	117+C
D		117+C	118+C
E		114+C	114+C
F		123+C	124+C
G		0	162+C
H		90+C	103+C
I		115+C	110+C
J		0	122+C
K		144+C	124+C
L		114+C	119+C
M		102+C	112+C
N		132+C	122+C

Positive controls: see Figure 8

Sterility controls: 1/80 vol of E=1, G=1, K=1, N=1

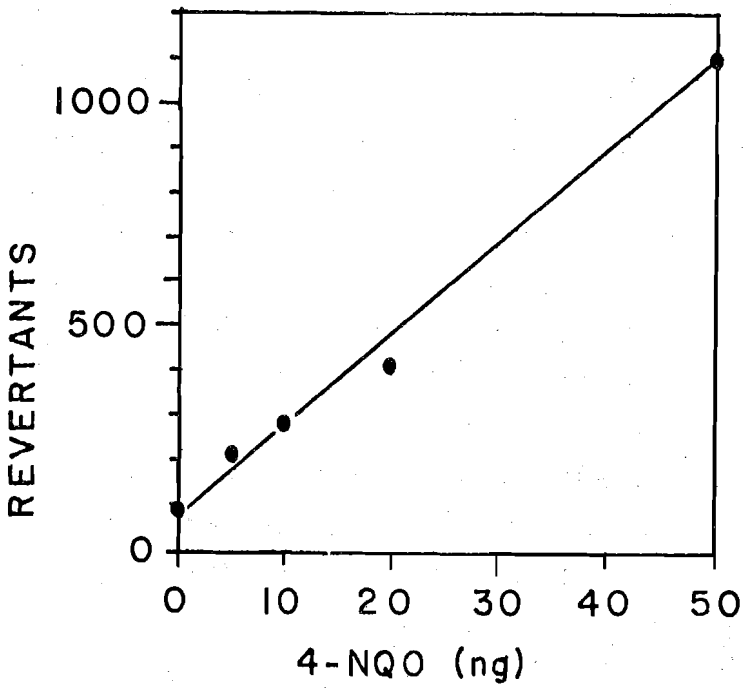

Figure 8. Response of TA100 to 4-NQO

Table 11. Recovery of 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide from Sangamon River Water

5 µg AF-2 added to 2 gal water (Fractions A-G) and (Fractions H-N); see text

Revertants of TA100 without S-9 mix			
Fraction	Vol extract: 0	1/8	1/80
A	92+C	96+C	110+C
B		101+C	114+C
C		0	119+C
D		128+C	115+C
E		128+C	115+C
F		112+C	116+C
G		428+C	124+C
H		139+C	111+C
I		100+C	141+C
J		0	141+C
K		132+C	130+C
L		111+C	111+C
M		159+C	118+C
N		348+C	135+C

Positive controls: see Figure 9

Sterility controls: 1/80 vol of B=1, C=2, J=4, K=6

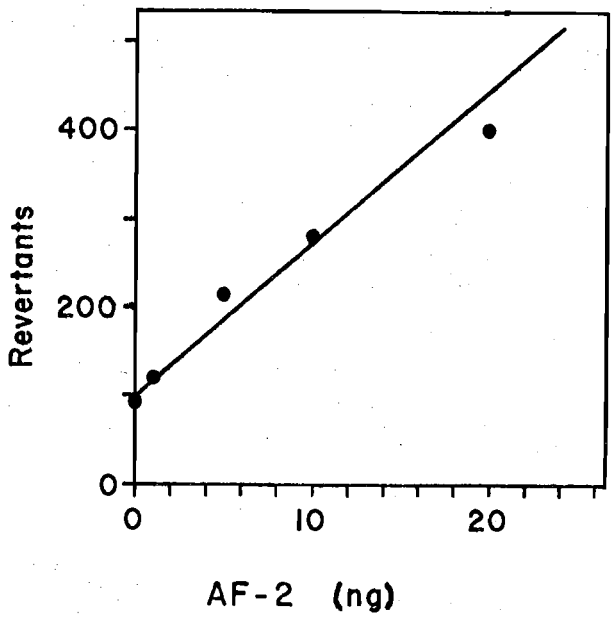

Figure 9. Response of TA100 to AF-2

and none to the other. The columns were processed, and mutagenic activity was detected in the expected fractions. The average recovery of AF-2 from these two columns, 2.8% (Table 13), is in agreement with AF-2 recovery from deionized water, 3.2% (Table 7). This result rests on the assumption that no naturally occurring mutagen was present in the neutral water-soluble fraction of the Sangamon River sample.

In a second experiment involving AF-2, 134 μg was applied to one of two columns in Boneyard Creek water (Table 12). The majority of the material was recovered in the neutral water-soluble fraction, as before. However, some activity was detected in all fractions, with appreciable amounts found in the acetone eluate of the IR 120 and IRA 400 fractions. The overall recovery was also improved to 9.9%.

The similarity of percent recovery of AF-2 from Sangamon River water and deionized water suggests that biochemical transformations are not so important a cause of loss of AF-2 as they appear to be for the other mutagens.

Overall this series of experiments suggests that it is possible to recover mutagenic activity from surface waters using the parfait/distillation method, even if the mutagen is initially present at ppb concentrations. However, it appears that the recoveries are strongly influenced by the stability of the compounds under the recovery and work-up conditions. The corollary of this conclusion is that only those naturally occurring mutagens stable under environmental conditions will be found by the method.

Naturally Occurring Mutagens in Illinois Surface Waters

The controls for the trial recoveries of mutagens from surface waters were assays of the same, but unspiked, surface water. Mutagenic activity was detected in two of these samples, the Salt Fork Creek and the Boneyard Creek which flows into the Salt Fork (Table 14). After this study was completed, the Illinois Environmental Protection Agency supported work to assay several surface-water samples by the parfait/distillation method; these assays are reported in Section III, Tables

Table 12. Recovery of 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide from Boneyard Creek Water

134 µg AF-2 added to 2 gal water (Fractions A-G)

Revertants of:		TA100 with S-9 mix			TA98 with S-9 mix		
Fraction	Vol extract:	0	1/8	1/80	0	1/8	1/80
A		136	836	209	25		
B			1328	406+C			
C			522	175			
D			380	212			
E			1768	378			
F			170	717			
G			0	1468			
H			138	132		26	34
I			154	152		42	23
J			139	145		33	36
K			179	192		40	37
L			169	148		43	30
M			140	156		28	33
N			212	149		36	30

Positive controls: TA98 with S-9 mix and 2 µg B(a)P=400
TA100 with 10 ng 4-NQO, without S-9 mix=586 (see Figure 10)

Repeat assays (fraction — vol):

B — 1/80 = 575, 1/150 = 473
F — 1/80 = 1385, 1/160 = 902
G — 1/80 = 1464, 1/800 = 617
No mutagen control = 312

A-J. In this series of assays, the Salt Fork Creek was again found to contain detectable mutagenicity. Mutagenicity was also found in the Fox River at Aurora, Illinois. In all, the Salt Fork Creek was sampled three times; it displayed mutagenicity twice, as reported in Tables 9 and G.2; it did not show mutagenicity once, as reported in Table 10. The two samples from Boneyard Creek have either displayed mutagenicity (Table 12) or not (Table 8).

Influence of Ionic Strength on Induced Mutagenicity

Tables 14 and J summarize the toxic and mutagenic activities recovered in the work performed respectively under sponsorship of the Water Resources Center and Illinois Environmental Protection Agency. The frequency with which toxicity was associated with the $TEACO_3$ eluate of the IRA 400 fraction (C or J) stands out in both tables. Recall that this fraction contains sufficient silicic acid to interfere with filter sterilization of the extract.

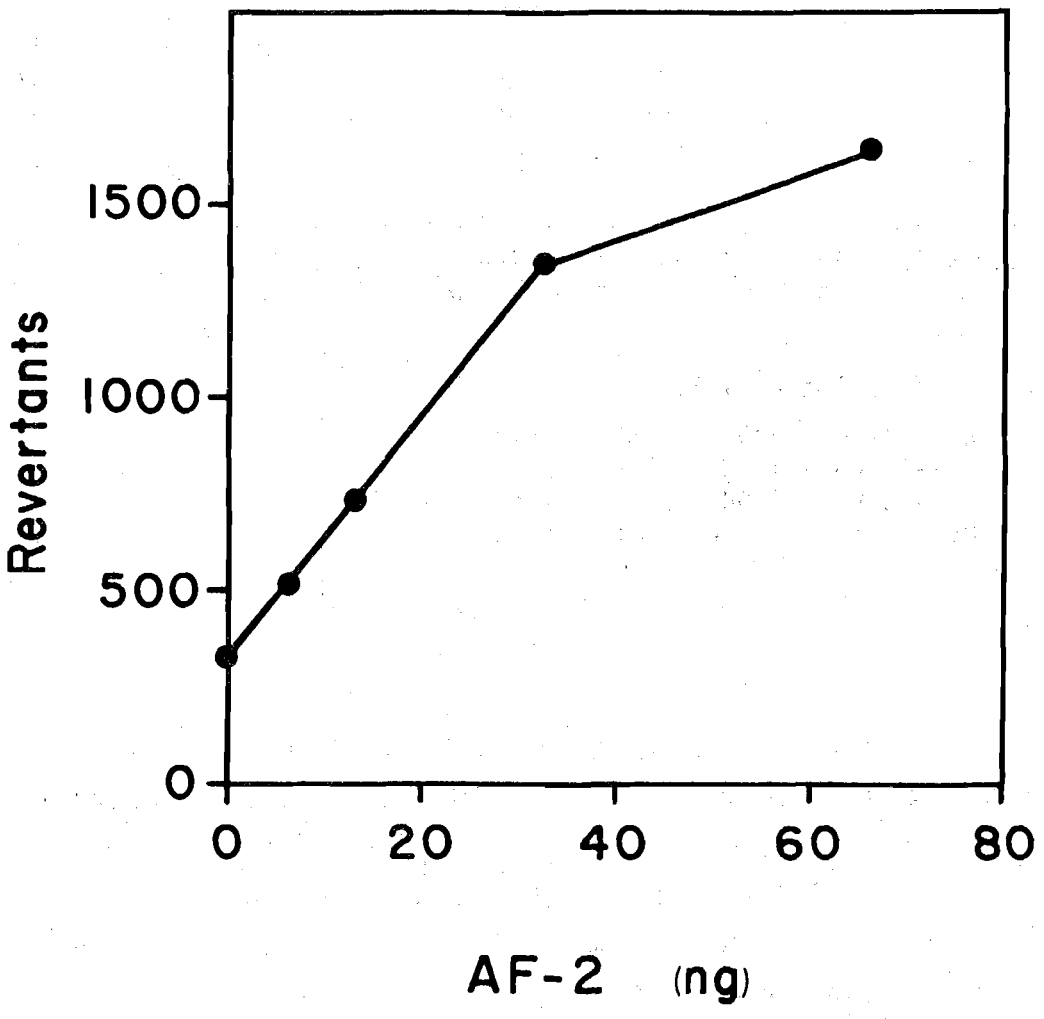

Figure 10. Response of TA100 to AF-2 and S-9 Mix

Table 13. Summary: Percent Recoveries of Mutagens from Environmental Water Samples

	% Recovery of Added Mutagen				
Fraction	B(a)P	EB	4-NQO	AF-2[a]	AF-2[b]
A	< 27[c]	<4	< 0.4	<0.9	0.19
B	< 27	< 4	<0.4	< 0.9	0.56
C	<27	< 4	< 0.4	< 0.9	0.08
D	72	20	< 0.4	< 0.9	0.05
E	< 27	312	< 0.4	<0.9	0.54
F	< 27	45	< 0.4	< 0.9	2.4
G	< 27	436	< 0.4	2.8	6.1
TOTAL	72	813	not detected	2.8	9.9

[a] Table 11. Assayed without S-9 mix; average of the recoveries from Fractions G and N.

[b] Table 12. Assayed with S-9 mix; recoveries based on repeat assays.

[c] The number following the < symbol is the detection limit of the assay, expressed as a percentage of the mutagen added to the water sample.

Table 14. Environmental Samples Showing Toxicity or Mutagenic Activity

Sample		Toxic Fractions	Mutagenic Fractions
Salt Fork Creek	(Table 9)	I,J	N
	(Table 10)	J	
Sangamon River	(Table 11)	J	
Boneyard Creek	(Table 8)	None	J

The second most frequent examples of toxicity were associated with $TEACO_3$ eluates of the IR 120 bed (Fractions B or I). Both of these extracts would be expected to contain salts which could significantly raise the ionic strength of the Ames assay top agars. Indeed, since each assay plate at 1/8 volume would contain the material recovered from 1 liter of water, a typical surface water having a conductivity equivalent to 5 mM NaCl could contain up to 5 mmoles of salt which would be added to the 2 ml of top agar. This could expose the bacteria and S-9 enzymes to equivalent molarities of 1-2 M.

To find out whether ionic strength alone could account for the observed inhibitions of reversion frequency, the experiment described in Table 15 was performed. The table shows that NaCl in amounts up to 2 mmoles did not adversely affect either the spontaneous reversion frequency of the bacteria or the ability of S-9 enzymes to activate B(a)P. Therefore it was concluded that the inhibitions observed in Fractions C and B of the surface-water extracts must involve some other deleterious attribute of the silica. The ionic strength or salt effects contributed by the silica might have a synergistic inhibitory effect on the assay.

Possible Artifact from Chromium Mutagenesis

The practice of removing silicate encrustations from the flasks used to vacuum distill Fractions G and N began only in the last part of the mutagen-spiking experiments. It became a routine practice when it was noticed that a faint green tint could be seen leaching from the encrustation into the sample during vacuum distillation. Suspecting that the color might be due to Cr^{3+} from the chromic-acid bath, adsorbed onto the silicate, the potential mutagenicity of Cr^{3+} was investigated. Venitt and Levy (1974) have reported that Cr^{6+}, but not Cr^{3+}, is a potent bacterial mutagen. It thus became imperative to determine both the activity of chromium under our assay conditions and the chromium content of our apparently mutagenic extracts of surface waters (Figure 11 and Table 16). Under our assay conditions, no mutagenicity was shown by Cr^{3+} in

Table 15. Influence of NaCl on Spontaneous and Induced Reversion of TA98 and TA100

Strain, mutagen	Average Revertants Per Plate			
	mmoles NaCl in top agar			
	0	0.5	1.0	2.0
TA100	138	149	141	122
TA100 with S-9 mix and 3 µg B(a)P	1024	1098	1044	1060
TA 98	23	28	36	40
TA 98 with S-9 mix and 3 µg B(a)P	384	468	430	384

amounts up to 5 µmoles; $Na_2Cr_2O_7$ by contrast was a potent mutagen in the range 20-300 nmoles but showed a very narrow range of activity. If present in excess of 500 nmoles, $Cr_2O_7^=$ inhibited even spontaneous reversion (Figure 11).

Realizing that the practice of washing all glassware in chromic acid created the potential for artifactual mutagenic response, we assayed available mutagenic water extracts for total chromium by neutron-activation analysis (Table 16). The results in the table are expressed in terms of the amount of chromium that would have been present in the highest volume of the extract tested for mutagenicity if all the Cr were $Cr_2O_7^=$. Comparing the result to the $Cr_2O_7^=$ mutagenicity dose response (Figure 11), it is evident that the mutagenicity found in the extracts of the Fox and Illinois Rivers and the Salt Fork Creek could not be accounted for by dichromate because too little chromium was present. The response of the Salt Fork sample (Table 9), however, could possibly be due to chromium. If all the Cr present were dichromate, the anticipated response of the sample would be toxicity. An appropriate mixture of Cr^{6+} and Cr^{3+} could have produced the response observed in Table 9 (dichromate is also mutagenic to TA98). Similarly, chromium could be responsible for the results in Table 11.

Table 16. Chromium Content of Extracts Showing Mutagenicity

Sample	Chromium, expressed as nmoles $Na_2Cr_2O_7$ per 2 ml extract
Lab deionized water	12
500 µM $Na_2Cr_2O_7$, sterile solution (used to construct Figure 11)	1181
Fraction N Table 9	302
Fraction N Table 11	642
Fraction B Table A	20
Fraction G Table G.2	50
Fraction G Table I.2	35

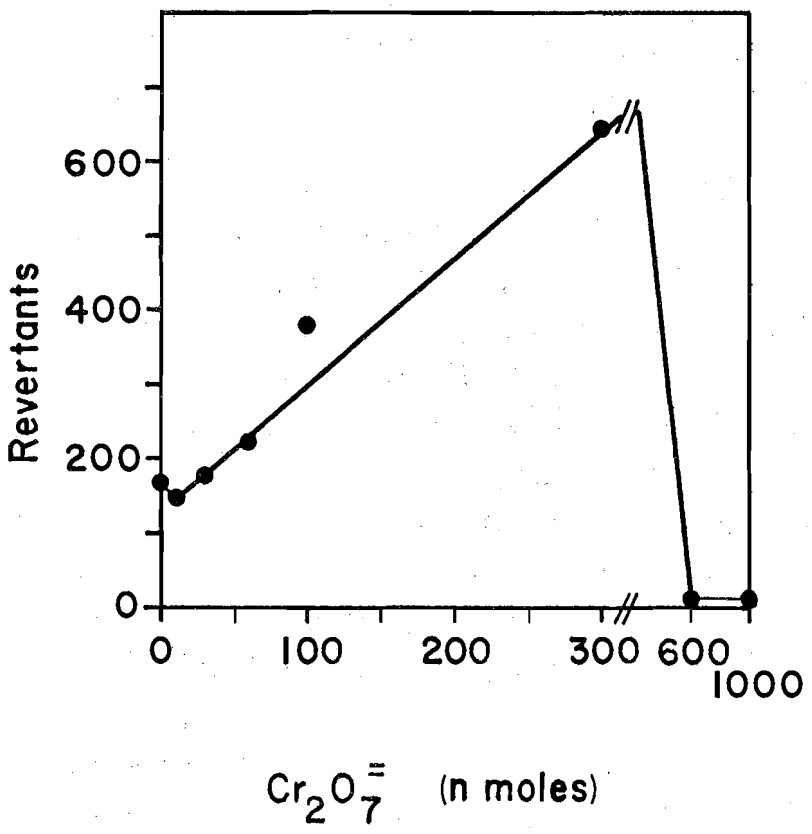

Figure 11. Response of TA100 to Dichromate

However, based on the responses seen in Tables 12 and G.2, the possibility that chromium induced some or all of the responses seen in Tables 9 and 11 does not alter the conclusions that natural mutagens occur in the Salt Fork (Table 9 compared to G.2) or that AF-2 can be recovered from environmental waters (Table 11 compared to 12). It is our opinion that the chromium detected in these samples is not contributing to the mutagenic response.

Finally, it can be seen in Section II that histidine in a sample can increase the spontaneous reversion frequency to levels that could be mistaken for a mutagenic response. Histidine would only be present in the $TEACO_3$ fraction from the IR 120 resin (Fraction B). Therefore, Fraction B from the extract of the Illinois River (the only mutagenic example of a B fraction, see Table A) was assayed for available histidine as described in Section II. The results of this assay revealed 0.23 μmole/ml available histidine in this extract. The neutral water-soluble fraction from the Fox River, by contrast, showed no detectable histidine in the same assay. Comparing the histidine levels in the Illinois River sample to the histidine-induced reversion of the Ames strains (Section II, Figure 12), it was concluded that all of the apparent mutagenicity of Fraction B from the Illinois River could be accounted for by the available histidine present in the extract—that is, no detectable mutagen was present.

CONCLUSIONS

The parfait/distillation method is able to recover some model mutagens from environmental surface waters, even from initial concentrations as low as 1 ppb. The extent of recovery is strongly influenced by the chemical and biological stability of the mutagen in the surface water, under the conditions of the recovery and work-up. Several of the model mutagens were apparently transformed during the work-up, some to toxic, nonmutagenic forms and one possibly to a more mutagenic form.

Analyses of Illinois surface waters, carried out during the validation studies and subsequently under contract to the Illinois Environmental Protection Agency, revealed significant mutagenic activity at 2 sites. In the Fox River at Aurora and the Salt Fork Creek at Urbana, the activity was found in the neutral, water-soluble fraction. Weak, potentially mutagenic responses were detected in the Illinois River at Peoria, in Lake Bloomington, and in the Embarras River near Lawrenceville.

The parfait/distillation method recovers neutral water-soluble mutagens, one of our primary research goals. It also recovers amphoteric compounds such as histidine. The recovery of anionic mutagens has not yet been demonstrated, and there is an interference with the assay of water-soluble, anionic compounds from the column. The interference is apparently due in part to the accumulation of silicic acid during fractionation.

The potential for artifactual results from histidine has been characterized, and a method to correct for this problem has been developed. The anticipated interferences in the Ames bioassay from the contribution of high ionic strengths (up to 1 M) by the extracts have been shown not to be significant.

Overall, the method represents significant progress toward a routine test for waterborne mutagens, but it requires further refinement before it can be implemented on a routine basis.

Significance and Relation to Water Resources Problems

Recent studies of drinking waters, drinking-water supplies, and chlorinated secondary effluents have revealed the presence of mutagenic compounds in these waters (Loper, Lang and Smith, 1978; Glatz *et al.*, 1978; Cumming, 1978). One study (Glatz *et al.*, 1978) showed that the process of treating water to prepare it for drinking apparently created mutagens, since extracts of the drinking water were mutagenic but extracts of the precursor drinking-water supply were not.

These findings point out the need for a rapid, simple test for mutagens in drinking waters and surface waters. Development of such a routine mutagen test was the objective of this research.

The procedure which has been developed differs from previous work in its emphasis on neutral water-soluble compounds and its use of a sample volume that is readily comparable to the volume consumed every few days by a normal person.

SECTION II

Factors Affecting the Reproducibility of the Ames *Salmonella* Reversion Assay

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ABSTRACT

The presence of histidine in complex, chemically undefined samples can lead to significant increases of spontaneous reversion frequency in the *Salmonella* reversion assay. The histidine present on an assay plate directly controls the number of divisions on the plate and therefore the final population of bacteria on the plate. Most spontaneous revertants arise during divisions on the plate. The frequency of spontaneous revertants of TA1535 and TA1538 is proportional to the final number of cells; the frequency of spontaneous revertants of TA100 and TA98 shows a biphasic response to added histidine. A simple assay for the determination of available histidine in complex samples is proposed, and conditions that enhance the survival of *Salmonella* inoculated on assay plates are described.

INTRODUCTION

Bacterial reversion assays are finding broad use in the screening of chemicals for potential carcinogenic activity. Recently such assays have been applied to complex, chemically undefined samples such as river water (Pelon, Whitman and Beasley, 1977), extracts of potable water (Glatz *et al.*, 1978; Loper, Lang and Smith, 1978), extracts of urine (Legator, Connor and Stoeckel, 1975; Yahagi *et al.*, 1977), and feces (Bruce *et al.*, 1977). Our laboratory has been concerned with the adaptation of the Ames *Salmonella* reversion assay to extracts of organic materials from surface waters.

We have studied the test with interest in those factors which cause systematic fluctuations that might be falsely interpreted as low-level, induced increases in reversion frequency. In this report, we have examined in particular the influence of histidine on the spontaneous reversion frequency of the *Salmonella* tester strains.

MATERIALS AND METHODS

Strains and Materials

Salmonella typhimurium TA100, TA98, TA1535, TA1538 and the mutagen 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2) were supplied by Dr. B.N. Ames. Strains were checked for histidine requirement, deep rough character, resistance to ampicillin and response to diagnostic mutagens, as recommended by Ames (Ames, McCann and Yamasaki, 1975). Frozen permanent stocks were prepared from 18 h L-broth (Lennox, 1955) cultures by adding sterile dimethylsulfoxide to a final concentration of 6% (v/v), freezing in dry ice plus acetone, and storing at -70°.

Assay of Spontaneous and Mutagen-Induced Reversion Frequencies

Inocula for assay of reversion frequency were grown 14 to 20 hours, shaking at 37° either in Difco Bacto nutrient broth supplemented

with 5 g/l NaCl or in Vogel Bonner medium E (Vogel and Bonner, 1956) supplemented with 0.4% glucose, 0.3 mM L-histidine, 5 mM D-biotin and 2% (^{v}/v) L-broth (enriched Vb). The culture was harvested by centrifugation (3,500 x g for 5 min) and resuspended by vigorous vortex agitation in one-half volume 0.9% NaCl. The density of this suspension was estimated from the A_{660} of a diluted sample measured in a Bausch and Lomb Spectronic 20 colorimeter using an 18-mm colorimeter tube. Based on previously constructed curves of L-agar-viable cells versus A_{660}, an A_{660} of 1.5 was assumed to be equivalent to 1 x 10^9 cells; the experimentally observed A_{660} of the diluted suspension was always between 0.10 and 0.50. (It is necessary to determine experimentally the equivalence between apparent absorbance and L-agar-viable count for each colorimeter used; the conversion used above applies only to our instrument.)

Based on this information, the suspension was diluted with 0.9% NaCl to a final calculated density of 1 x 10^9 bacteria/ml and held on ice. For experiments in which the inoculum was varied, bacteria were resuspended in 25% of the original volume; and suspensions of approximately 1 x 10^{10}, 1 x 10^9, and 1 x 10^8 bacteria/ml were prepared by dilution, with reference to the A_{660} as described. To estimate reversion frequency, 0.10 ml of the 1 x 10^9 bacteria/ml stock (hereafter called the basic stock) was delivered from an automatic pipet into 2 ml of top agar supplemented with histidine and biotin as described in this section. It is necessary to agitate the tube of basic stock before each removal of bacteria; if not agitated, the cells settle perceptibly over the course of hours. The top agar was quickly mixed by vortex agitation and plated on a Vb basal agar containing 2% glucose. Following incubation at 37° for 72 hours, the His^+ revertant clones were counted.

Viability on L-Agar Versus Vb Agar

For each experiment, the viability of the basic stock was

determined on L-agar (LA) and on Vb agar supplemented with 2% glucose, 80 nmoles D-biotin, and 5 μmoles L-histidine (VbA). The basic stock was diluted serially 10^{-6} in 0.9% NaCl, and duplicate 0.20 ml aliquots were plated either in a top agar or by sterile spreader.

Histidine-Limited Growth Yield of Unknowns

Histidine-limited growth yield was estimated from increases in A_{660} of liquid cultures having histidine in concentrations from 0 to 50 nmoles/ml and excess biotin. A single large culture of the histidine auxotroph was prepared by dilution of basic stock into Vb liquid medium containing 0.4% glucose and 5 mM biotin to an initial A_{660} of approximately 0.01. Replicate 5 ml aliquots of this culture were distributed into a series of sterile 18-mm colorimeter tubes. Each tube was then supplemented with histidine and 0.9% NaCl to bring the final volume to 5.5 ml and the final concentration of histidine to 0, 10, 20, 30, 40, and 50 nmoles/ml. Sterile extracts of surface waters were added to the remaining tubes, in amounts of 0.50 ml and 0.05 ml plus 0.45 ml NaCl. The cultures were incubated shaking at 37°. The stationary phase A_{660} of the standards was plotted versus concentration of histidine to make the standard curve. The amount of available histidine in unknowns was determined from the stationary phase A_{660} with reference to the standard curve.

RESULTS AND DISCUSSION

Influence of Histidine and Inoculum Size on Spontaneous Reversion Frequency

Figure 12 shows the response of spontaneous reversion frequency to added histidine. Strains TA1535 and TA1538 show proportional increases over the entire range of histidine, whereas the strains carrying plasmid pKM101 exhibit a biphasic increase. In these R-factor-bearing strains, the increase of His^+ revertants rises

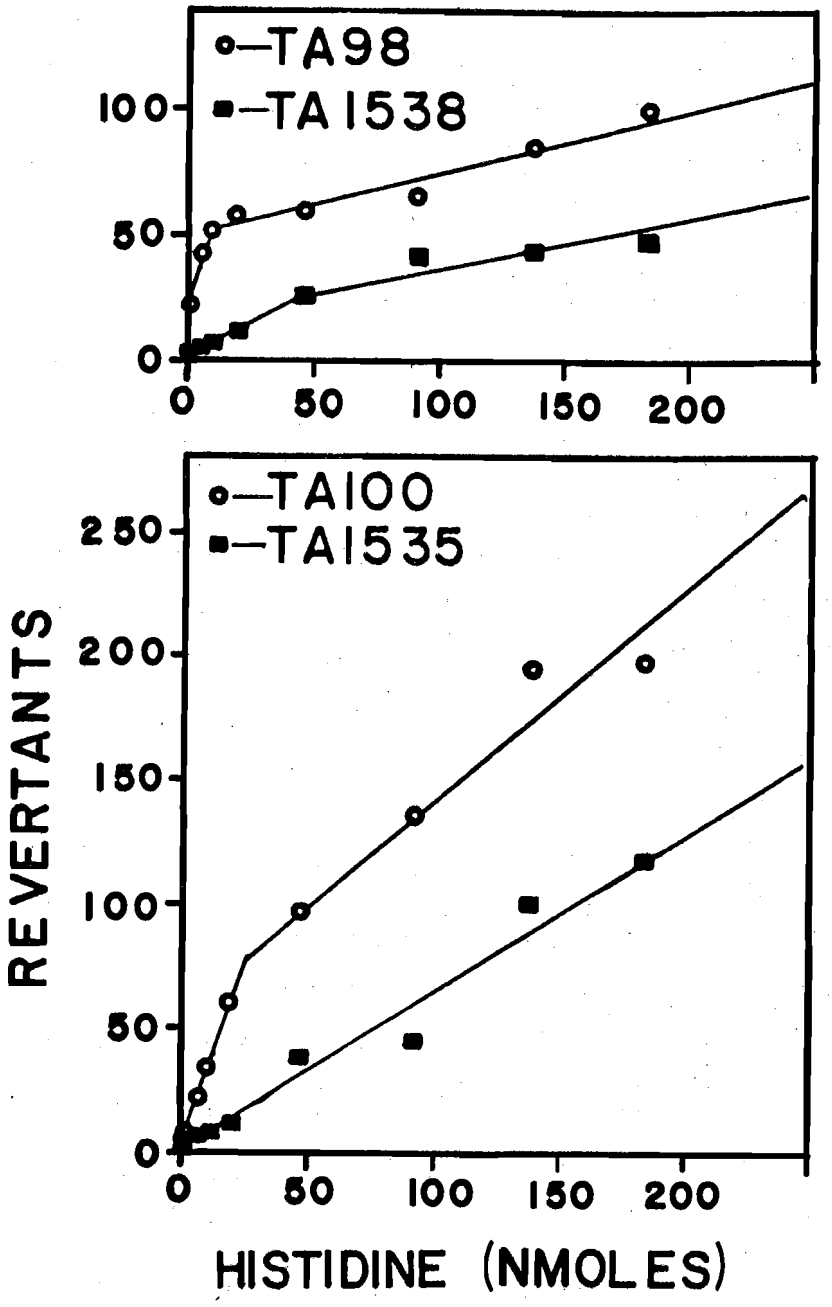

Figure 12. The Effect of Added Histidine on Spontaneous Reversion Frequency

Strains grown 18 h in Vb liquid supplemented as described in Table 17 were twice washed by resuspension in 0.9% NaCl, and 1 x 10^8 cells were plated as described by Ames, McCann, and Yamasaki (1975) except the top agars contained the indicated amount of histidine. Each point is the average of three plates.

steeply in the range 0-50 nmoles histidine and then approximately parallels the curve for the corresponding strains without R-factor.

Figure 13 shows that relatively large variations in the number of bacteria inoculated on a plate were required to affect the spontaneous reversion frequency. The dominant influence was not the inoculum size but the amount of histidine added. Addition of 0, 10, or 100 nmoles histidine produced roughly proportional increases in spontaneous reversion frequency at each level of inoculation. Increase in reversion frequency induced by 2 μg sodium azide was also more strongly dependent upon the amount of histidine added than on variations in the inoculum size.

Mortelmans and Stocker (1976) have performed similar experiments comparing glycerol-grown *Salmonella typhimurium his G 46* with or without plasmid R46. R46 is the progenitor of pKM101, the plasmid found in TA100 and TA98. They found that the spontaneous reversion frequency was proportional to added histidine and that inoculum size had little effect on reversion frequency in the presence of 60 nmoles histidine. They did not observe the biphasic response seen in Figure 12.

Under the conditions of the reversion assay, growth on the plate becomes limited by a lack of histidine for all strains but stable revertants which have both adjusted to the culture conditions and regained the ability to synthesize histidine from glucose. It seems reasonable therefore to interpret the systematic variation of spontaneous reversion frequency with added histidine in terms of the number of new cells arising on the plate. If the number of new cells is a linear function of the amount of histidine added, and if each newly arising cell has a fixed probability of being revertant, then the frequency of spontaneous reversion should be a linear function of added histidine. Similarly, for any given level of inoculation,

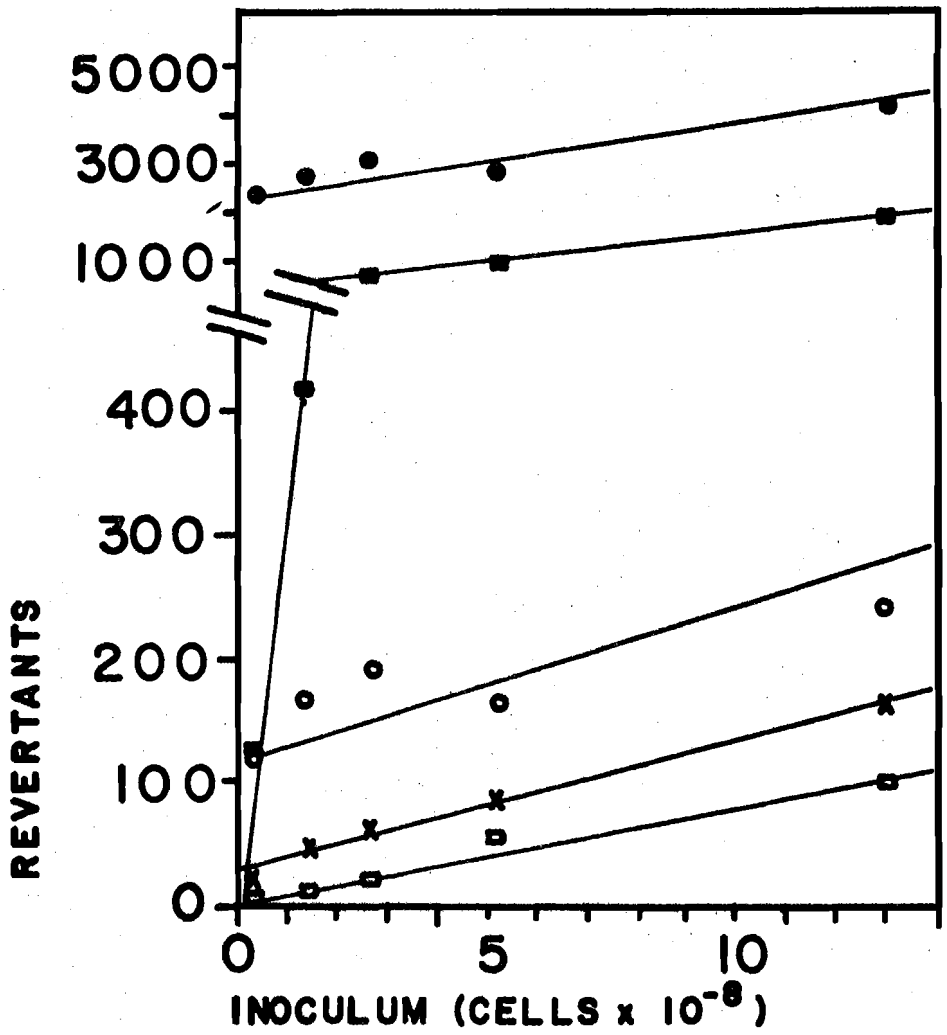

Figure 13. The Effect of Inoculum Size on Spontaneous and Azide-Induced Reversion Frequencies

Strain TA1535 was grown 18 h in Vb liquid supplemented as described in Table 17, centrifuged, resuspended in 0.9% NaCl, and plated in top agars containing excess biotin and 0 (□), 10 (×), or 100 (○) nmoles histidine. Samples containing 2 μg NaN_3 were plated with 0 (■) or 100 (●) nmoles histidine. At the time of plating, inoculum size was estimated from turbidity, with reference to a standard curve. The actual viable count was determined after diluting in 0.9% NaCl and plating on L-agar. Each point is the average of three plates.

the spontaneous reversion frequency should be the sum of pre-existing revertants in the inoculum plus the revertants arising during division on the plate. The results shown for TA1535 and TA1538 in Figures 12 and 13 are consistent with this interpretation.

Histidine-Limited Growth Yield

To test whether the amount of added histidine produced proportional increases of viable cells, we attempted to recover bacteria from the top agar. This approach was abandoned when we found that the reproducibility of the recovery was poor in reconstruction experiments, even recoveries from freshly poured top agars. Experiments in liquid culture were undertaken then to simulate conditions in the top agar (Figure 14). First the relationship of A_{660} to LA-viable cells was established.

The ratio of A_{660} to LA-viable cells was found to be constant $\pm$20% for all strains in both histidine-limited and control (excess histidine) cultures during both exponential and stationary phases. The LA-viable titer and A_{660} of the stationary-phase cultures remained constant for over 20 hours (Figure 14, panel A). After more than 20 hours, the A_{660} and titer increased as His^+ revertants took over the culture.

Knowing that the increases of A_{660} shown in the figure reflected proportional increases in titer, it was possible to calculate the growth yield for histidine by replotting the maximum A_{660} versus nmoles of histidine added (Figure 14, Panel B). 1×10^2 nmoles histidine were found to produce 1×10^9 cells, in reasonable agreement with the value of 5×10^9 nmoles histidine found with *Salmonella typhimurium his G 46* grown in glycerol (Mortelmans and Stocker, 1976).

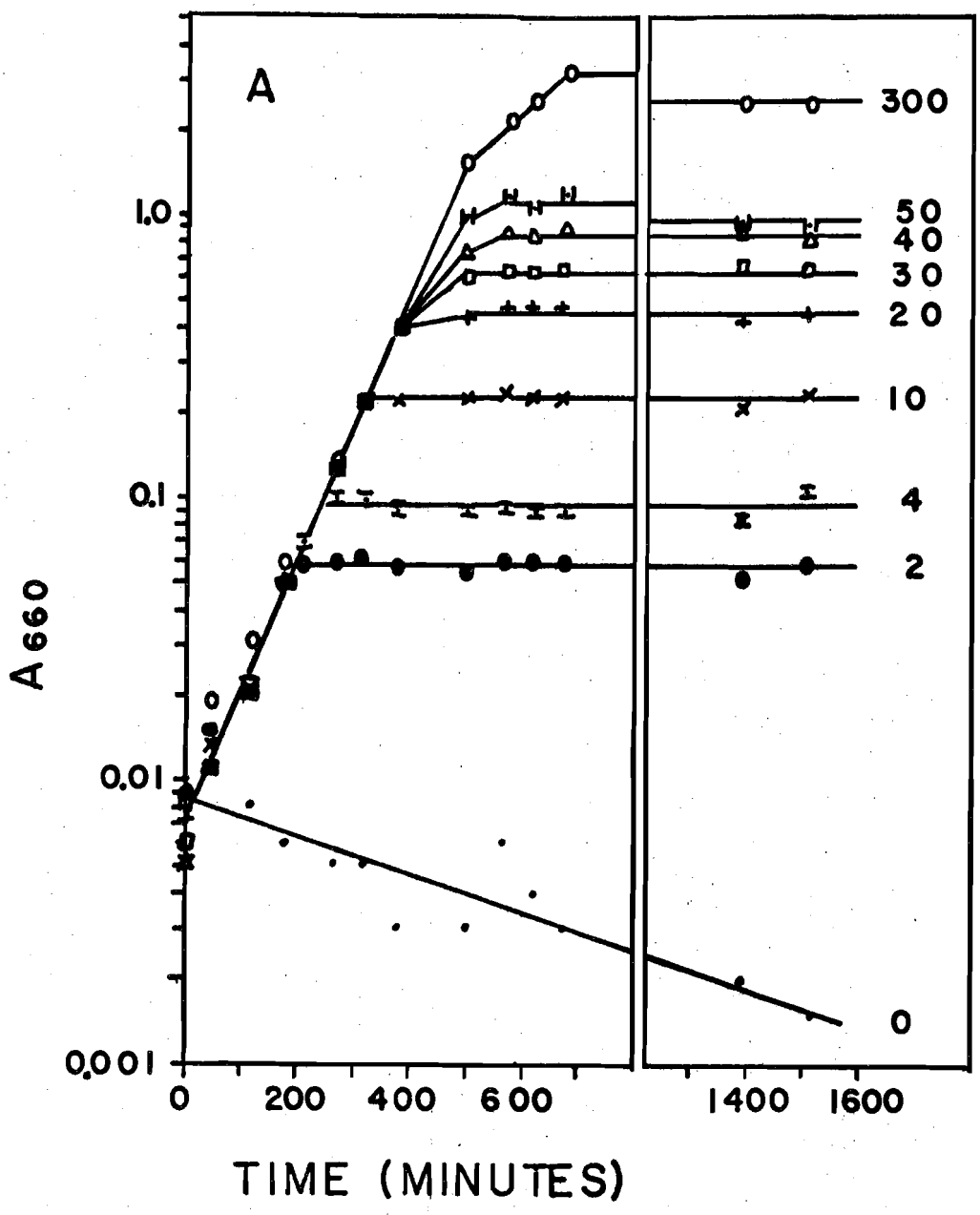

Figure 14. Histidine-Limited Growth in Liquid Culture

A. The A_{660} versus time of TA100 grown in Vb liquid medium supplemented with excess biotin and limiting histidine (see Methods). The numbers to the right of each curve give the initial concentration of added histidine in nmoles/ml.

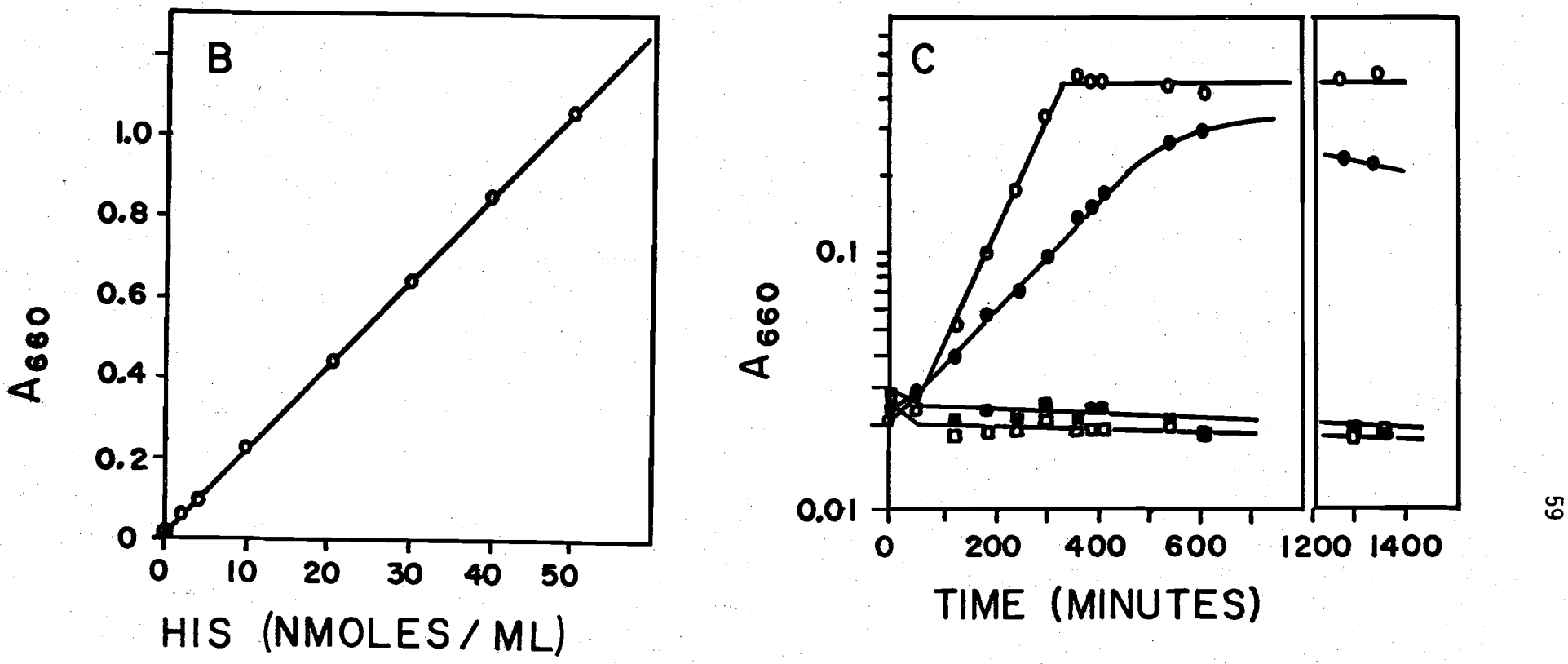

Figure 14 (B and C). Histidine-Limited Growth in Liquid Culture

B. A replot of the maximum A_{660} seen in A versus concentration of added histidine.

C. The A_{660} versus time of TA100 supplemented with no histidine (squares) or 20 nmoles/ml histidine (circles), in the presence (filled symbols) or absence (open symbols) of 500 ng AF-2.

Reversion Rate of R-Factor-Bearing Strains

Within experimental error, the histidine growth yield was the same for all strains. Therefore the differences in reversion frequency seen when comparing TA100 to TA1535 and TA98 to TA1538 in the range 0-50 nmoles histidine must reflect different rates of reversion, i.e., revertants per division, in the R-factor-bearing strain compared to the corresponding strains without R-factor. With the addition of more than 50 nmoles histidine, the curves for the R-factor-bearing strains became approximately parallel to those of their plasmidless parents. We therefore conclude that the R-factor effect on reversion rate is transient, operating only during the initial division(s) on the plate.

The influence of histidine on R-factor enhancement of spontaneous reversion rate might operate through any of several mechanisms. It is known, for example, that the rate of UV-induced reversion of His^- ochre and frameshift mutations is elevated by increasing the rate of transcription of the histidine operon (Savic and Kanazir, 1972). Since the inoculum used in the experiment described in Figure 12 was washed and resuspended in histidine-free medium, it is likely that the histidine operon of these cells was derepressed prior to plating, thus elevating the rate of transcription of the histidine operon. But this elevation alone would not account for the observed behavior of the spontaneous reversion frequency since the plates receiving no histidine would have been the most strongly derepressed, yet they displayed the lowest spontaneous reversion frequency.

Histidine limitation would have the additional effect of limiting net protein synthesis. Walker (1977) has studied the pKM101 enhancement of spontaneous reversion frequency in *E. coli*; he has concluded on the basis of studies with a *tif* mutant that the plasmid effect interacts synergistically with a *tif*-controlled mutator activity. The *tif*-controlled activity is inducible and requires

protein synthesis for expression (Witkin, 1969).

The behavior of TA100 and TA98 under severe histidine limitation could be rationalized if starvation for histidine induced transcription of a gene (encoded on chromosome or plasmid) that was analogous to the *tif*-controlled mutator. Induction would occur as a result of amino-acid starvation, but synthesis of the protein product required to elevate reversion rate would be limited by the amount of histidine supplied in the range 0 to 50 nmoles histidine. Above this level, sufficient histidine would be available to permit synthesis of the normal amounts of the protein, but this level of histidine would also relieve the starvation that led to induction. Therefore, only in the initial division would the enhanced reversion rate be observed, as the messages synthesized during amino-acid starvation were translated.

In stringent bacteria, amino-acid starvation causes synthesis of highly phosphorylated nucleotide signals, including ppGpp and ppApp (Cashel, 1969; Rhaese and Groscurth, 1976). *E. coli* bearing the *tif* mutation respond to exogeneous adenine at permissive temperatures in exactly the same manner as they do to elevated temperature (Kirby, Jacob and Goldthwait, 1967). Exogeneous guanosine and cytidine counteract both temperature-induced and adenine-induced filamentation and prophage induction in this strain. Therefore it is possible that a nucleotide signal may be involved in the postulated induction of mutator activity by amino-acid starvation.

Histidine and Reversion Assays

The increase of spontaneous reversion frequency caused by histidine has direct consequences for the reproducibility of background reversion frequency between experiments and for the detection of low levels of mutagens in chemically undefined samples. In the protocol used by Ames, 91 nmoles of histidine were added in the top agar and an unknown amount was added in the 0.10 ml of spent nutrient broth (Nb) that

accompanied the inoculum (Ames, McCann and Yamasaki, 1975). We have assayed the amount of histidine available in aliquots of spent filter-sterilized Nb by estimation of the amount of growth of histidine auxotrophs that it will support in comparison to growth supported by known amounts of histidine (Figure 14). Ten to thirty nmoles of histidine were found in various batches of spent Nb, representing a possible variation of background reversion frequency of about 15%. This variation is not unacceptable, but is is one that is easily controlled by harvesting the bacteria and resuspending in a histidine-free medium before inoculation of the plates.

Histidine in chemically undefined samples represents a more serious concern, especially if one wishes to detect weak mutagens or small amounts of mutagens. Ames has mentioned the need to estimate and correct for histidine in extracts of urine (Yamasaki and Ames, 1977). In samples of environmental origin such as surface waters, careful control for histidine is particularly important since the content of mutagens may be expected to be low.

A recent paper reports small but statistically significant increases in reversion frequency induced by Mississippi River water (Pelon, Whitman and Beasley, 1977). From Figure 12, it can be seen that increases of more than twofold can easily be induced by histidine in the absence of mutagen. The highest induced increases reported in the Mississippi water study could be accounted for if the water contained 20 nmoles per ml (20 μM) histidine.

There is too little quantitative information in the literature on the histidine content of fresh water to indicate whether the results of this study represent histidine or mutagen. Histidine has been qualitatively detected in pond water (Litchfield and Prescott, 1970), in estuaries similar to part of the area sampled in the Mississippi study, and in marine waters. In the estuary of Virginia's

York River, 5 nM free histidine has been found (Hobbie, Crawford and Webb, 1968). Histidine is present in detritus from *Spartina* marsh grass, which is the characteristic vegetation present in the final 100 miles of the Mississippi River, including several sites sampled in the study (Hall, Weimer and Lee, 1970). Concentrations of histidine in marine waters vary widely: from trace to 34 nM free; 19 to 460 nM dissolved, released by acid hydrolysis; and 18 to 70 nM particulate, released by hydrolysis (Siegel and Dengens, 1966). Low concentrations of histidine should be expected in waters receiving effluent from sewage treatment. Randkte *et al.* (1978, p.207) report the concentration of soluble histidine in effluent from an activated-sludge unit to be 80 nM. A normal 70-kg human excretes daily 69-460 mg total histidine in urine and 98-147 mg in feces (Hunter, 1971).

From this information it seems unlikely that histidine was present in the Mississippi samples in μM concentrations. However, we have been unable to find quantitative information on the histidine content of any river water. Histidine concentration becomes important in our studies of surface waters because we, like others (Glatz *et al.*, 1978; Loper, Lang and Smith, 1978), concentrate the organic material obtained from the original water several hundredfold.

Estimation of Histidine in Complex Mixtures

Any assay used to estimate the content of histidine available to the *Salmonella* tester strain should mimic as closely as possible the conditions of the reversion assay. For example, straightforward chemical analysis of free histidine may underestimate available histidine if the content of histidine-containing oligopeptides in the sample is high. Conversely, acid hydrolysis of the sample may liberate histidine from sources such as protein which might not provide all of this histidine to the bacteria under the conditions of the assay. Estimation of the amount of growth supported by an

unknown sample is a simple way to determine the amount of histidine actually available to the bacteria in the unknown sample under the conditions of the reversion assay. Environmental samples containing particulate matter or material absorbing at 660 nm can pose problems, however. We normally sterilize unknowns by filtration through 0.2-micron Nuclepore filters, which solves the former problem. If the color in a sample is not too intense to mask increases in turbidity, it can be assayed as illustrated in Figure 14. To insure that the changes in A_{660} observed in colored samples actually correspond to increases in bacteria rather than to changes in coloration, at the end of the experiment the A_{660} of the sample should be read after bacteria have been removed by centrifugation and filtration.

Panel C of Figure 14 illustrates the behavior of cultures containing both histidine and mutagen. Even the presence of mutagen, no increase of A_{660} occurred unless histidine was present. In the presence of histidine the mutagen induced filamentation of the bacteria, which was observed in a phase contrast microscope. Filamentation caused the rate of increase of A_{660} to be lower than the rate of increase seen in the standards, and the final A_{660} was slightly lower than that of the standard having the same amount of histidine. A net increase of A_{660}, therefore, indicates the presence of histidine in the sample; the deviation of the A_{660} increase from the exponential part of the control curves indicates the presence of a toxic, potentially mutagenic agent in the sample. The significance of such a result depends upon the reversion frequency induced by that sample. In general we have found that histidine alone can induce a maximum increase of only fourfold over the reversion frequencies normally found for the Ames strains. Unknowns that induce greater reversion frequencies must contain mutagen.

In deciding whether an environmental sample contains mutagen, we assay only for histidine and correct for histidine-induced increases

of spontaneous reversion frequency in the samples that show a one- to fourfold increase over the control frequency. To calculate the histidine-induced increase, we add to the experimental reversion frequency of the control (no mutagen) the product of the concentration of histidine-like activity in the sample and the slope of the appropriate curve of revertants versus nmoles histidine shown in Figure 12, using the slope in the range 50-250 nmoles histidine. The difference between this sum and the experimentally induced reversion frequency is taken as the increase due to mutagen in the sample. Our experience so far with extracts of surface waters has been that unknowns either contain sufficient histidine to account fully for the observed increases of reversion frequency or they contain no growth-supporting activity (histidine) at all.

Viability of Strains under Assay Conditions

We found two factors unrelated to histidine that may affect the quantitative response of the Ames reversion assay. During study of the ratio of A_{660} to viable count, it was found that the viability of the strains grown on VbA was less than that on LA. Furthermore, the ratio of viability on VbA compared to LA was lower if the strains were grown in liquid Nb with NaCl, as recommended by Ames, than if they were grown in supplemented Vb liquid medium (see Table 17). In the experiment described in the table, the Nb or Vb liquid cultures of each strain were diluted in 0.9% NaCl at room temperature, and duplicate platings of about 4×10^2 bacteria were made from the same final dilution tube onto VbA and LA. The ratio of A_{660} to LA-viable cells was always constant within experimental error; VbA viability was more variable but was always lower than the LA viability—typical values are shown in the table. No significant differences were seen when comparing plating by sterile spreader to plating in top agars.

Table 17. Relative Viability of Strains Grown in Enriched Vb or Nb Liquid Medium when Plated on Vb Agar Versus L-Agar (Plating Shock)

Liquid growth medium [a]	Viability on Vb agar/L-agar [b]			
	TA100	TA1535	TA98	TA1538
Nb plus NaCl	0.01	0.97	0.006	0.006
Enriched Vb	0.93	0.98	0.22	0.23

[a] Inocula were grown 18 h in Nb containing 0.5% NaCl (Ames, McCann and Yamasaki, 1975) or in Vb liquid supplemented with 0.4% glucose, 0.3 mM L-histidine, 5 µM D-biotin, and 2% (v/v) L-broth.

[b] Following dilution in 0.9% NaCl, bacteria were plated on Vb agar supplemented with 5 µmoles L-histidine and 80 nmoles D-biotin and on L-agar (see text).

We refer to this decrease in viability on minimal plates as a "plating shock." In the presence of histidine on the plate, such a decrease in viability of the inoculum would have little effect on spontaneous reversion frequency or on the final amount of bacteria arising on the plate (Figure 13). However, since the growth of bacteria on the plate requires time, it is possible to predict that very short-lived mutagens will show a lower induced reversion frequency if the initial inoculum has a low ratio of VbA to LA viability compared to assay conditions with a higher VbA/LA-viability ratio. The effective target for such a mutagen would be only the initial viable cells in the inoculum, not the cells arising on the plate at later times. Witkin (1956) has described a decrease of revertant yield in untreated *E. coli*, associated with the shift from Nb to a minimal reversion-assay medium. In her work, growth of the inoculum

in the same medium (supplemented with the required amino acid) that is used to measure the reversion frequency significantly enhanced the yield of revertants. For these reasons we routinely culture bacteria to be used for reversion assay in the supplemented Vb liquid medium described in the table.

Finally, we have found that the use of DMSO in amounts greater than 50 µl can strongly affect the induced revertant yield, even though the spontaneous reversion frequency is not strongly influenced by DMSO up to 0.5 ml per plate. We have observed both increases and decreases, occasionally up to 70%, of induced reversion frequency caused by excess DMSO. Yahagi *et al.* (1977) have reported a similar finding during exposure of bacteria to N,N-dimethylnitrosamine in DMSO.

SECTION III

Results of Mutagenicity Assay of Illinois Surface Waters Using the Parfait/Distillation Recovery Method and the Ames Assay

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INTRODUCTION

Recent studies of the composition of trace organic materials in U.S. surface waters have detected the presence of mutagenic chemicals (Glatz *et al.*, 1978; Loper, Lang and Smith, 1978; Simmon, Kauhanen and Tardiff, 1977; Cumming, 1978). Mutagens are chemical or physical agents able to damage the genetic material of living cells, the DNA. DNA is the material which encodes the information ultimately reponsible for the normal functioning of each cell in an organism. Damage to DNA can lead to alteration of this information when the damage is improperly repaired. This is called a mutation. Mutations can manifest themselves in an organism as a cancer, as birth defects, as genetic diseases such as hemophilia and mongolism, or as a very slow and subtle degeneration of the vitality of the human species. The presence of mutagens in surface waters is therefore a cause for concern. At present it is difficult to quantitate the degree of hazard posed by mutagens in surface waters, partly because the extent of water pollution by mutagens is unknown.

Only the very first studies attempting to detect mutagens in water are completed. These studies have found significant levels of mutagenic activity in approximately 20% of the surface waters surveyed, mainly in Iowa and Illinois (Glatz *et al.*, 1978; and this report). Of greater concern is the finding in Iowa that about 70% of the finished drinking waters assayed contained mutagens even though the untreated source water was free of detectable mutagenic activity.

Clearly, it would be desirable for regulatory agencies to have available a simple test for the presence of mutagens in water, both surface waters and finished drinking water. The development of such a test was begun two years ago in the laboratories of the Institute for Environmental Studies, University of Illinois at Urbana-Champaign. Based upon the results of a major USEPA study, "Monitoring to Detect Previously Unrecognized Pollutants" (Ewing *et al*, 1977, p. 32), the test-development effort focused upon the recovery and detection of nonvolatile mutagens, assaying for mutagenicity with the widely used Ames *Salmonella*/microsome assay. The recovery of organic compounds was accomplished by the passage of the water sample through a series of adsorbent beds (silica gel, cation-exchange and anion-exchange resins) followed by concentration of the material in the effluent under vacuum. All of the beds were contained in a glass and teflon column, called the parfait column. Recovery of compounds from each bed of the parfait column was accomplished with both an ionic eluant, 2 M triethylammonium carbonate ($TEACO_3$), and an organic eluant, acetone. These eluants were removed from the sample under vacuum, and the residue was applied to the Ames assay following filter sterilization. This procedure has been described in detail in Section I.

The parfait/distillation recovery method has been subjected to a limited number of experiments to validate its ability to recover known mutagens spiked into environmental waters. As a part of a major Illinois EPA study of organics in waters and effluents, the IEPA supported the assay of several Illinois surface waters by the parfait/distillation method. Ten mutagenicity assays were completed. This report presents the results of that study.

METHODS

All of the methods employed are described in Section I. Section I also describes the format for reporting data and gives a brief discussion of the criteria for determining significance of the results.

SELECTION OF SITES

In general, waters were chosen for study because they were drinking water supplies or because they received significant effluent discharges. Table 18 lists the sites sampled.

RESULTS AND DISCUSSION

The data from the mutagenicity assays of the Illinois surface waters are presented in Tables A through I; the toxic and mutagenic responses obtained are summarized in Table J.

To be cited as a significant toxic response in Table J, the sample tested had to induce a response less than one half the corresponding control; samples inducing a number of revertants greater than 2 times the corresponding control were cited in the table as significantly mutagenic. A third category of marginally positive mutagenic responses has also been included in the table. It includes samples that induced reversion frequencies greater than 1.8 times control but less than or equal to 2 times control. By Ames' criteria they cannot be considered significantly positive results. However, because of the relatively small random variability in our modification of the assay and the small volume of sample represented by each assay plate at highest dose, i.e., approximately 1 liter of sample, these marginal results are reported separately.

The toxic responses summarized in Table J occur predominantly in Fraction C, the $TEACO_3$ eluate of the IRA 400 resin. A discussion of this finding is found in Section I. Also included in Section I is a discussion of potential artifactual results from contamination of the sample by dichromate ion carried over from the chromic-acid bath used to clean the glassware.

The results of the mutagenicity assay of the sites sampled included two parallel sets of assays of the same water, one from Lake Decatur (Table D) and the other from secondary effluent from the Peoria sewage-treatment plant (Tables B and C). These parallel sets of assays were attempts to test the reproducibility of the parfait/distillation method. In the Decatur series, duplicate samples were taken and the parallel samples were fractionated and worked up simultaneously. One fraction, G, was lost. The mutagenic responses of the remaining parallel fractions (Table D) were very similar, except for Fraction C compared to Fraction J. In no case did a significant mutagenic response appear in either set of fractions.

Two samples were collected in the Peoria treatment-plant effluent series, but only one was fractionated immediately (Table B). The second was stored for 29 days at 4° before fractionation to permit possible formation or loss of mutagens during storage (Table C). The treatment-plant effluent was chosen since we expected it to offer the best possibility of reactive generation or loss of new compounds due to the presence of organic material and residual chlorine in the sample. Note that each sample consisted of only one gallon of material rather than two because of the large amount of flocculant material in this sample. Again, no significant differences were found between the two parallel samples, except perhaps for a decrease in the toxic potential of Fraction C on standing.

The results of the mutagenicity assay of the other sites studied included two significant mutagenic responses. The Fox River, sampled at Aurora, Illinois (Table I, Fraction G), showed the strongest positive response recorded from the entire study. In the initial assay of the Fox River samples, the positive control for TA100 did not show a significant increase, although Fraction G did. Also in that assay (Table I.1), the sterility controls were unacceptably high. Following refiltration, the sample was assayed (Table I.2). Again, strong mutagenicity was seen in Fraction G, this time with acceptable positive and sterility controls. The sterility controls will be discussed later. The Fox River result is notable because of reports of high frequencies of tumors in fish caught in this river (Brown *et al.*, 1977).

The other significant mutagenic response was seen in the Salt Fork Creek composite sample (Table G). Here also, the positive controls were unacceptable in the initial assay, but the repeat assay showed normal controls and significant mutagenicity in Fraction G. The Salt Fork and the Boneyard Creek which flows into it have been assayed several times, showing a mutagenic response three out of the five times.

Assay of the Illinois River at Peoria (Table A) showed significant mutagenic activity in Fraction B with both TA98 and TA100. Fraction B is the $TEACO_3$ eluate of the IR 120 resin and therefore should contain any histidine present in the water sample. As described in Section II, histidine can lead to an artifactual response due to additional histidine-supported growth of the indicator bacteria on the assay plate, giving rise to increased numbers of spontaneous revertants. This type of response is characterized by a similar percentage increase of revertants of both TA98 and TA100, since both respond to histidine even though these strains respond differentially to mutagens. To estimate the amount of histidine in the sample, a growth-limitation assay was performed as previously described. The results showed that Fraction B contained 0.23 μmoles of available histidine, an amount sufficient to account for both the TA100 and TA98 responses. Therefore, it was concluded that this fraction did not contain detectable mutagenic activity. It should be added that the Fox River Fraction G has been assayed by the histidine-limitation assay and found to contain no detectable histidine.

The results from the sterility controls from a number of assays described in the data present a serious problem of interpretation. (A sterility-control plate consists of the S-9 mix and sample without *Salmonella.*) In Tables A, D, E, F, H, and I, one or more values of the sterility controls indicate that contaminants in the sample should induce an apparent reversion frequency greater than the actual reversion frequency observed. For instance, the Table H.1, 1/80 volume of Fraction D showed 49 contaminating colonies on the sterility-control plate. When 1/8 volume of D was applied to the reversion assay, only 78 colonies appeared (TA98) rather than the 490 expected to appear based on the sterility control. In Tables F, H.1,

and I.1, there are even samples where more colonies appeared on the sterility-control plates than on the reversion-assay plate receiving the same volume of sample. Consider Table I.1, in which 1/80 volume of G showed 912 colonies on the sterility-control plate but only 168 (TA100) or 54 (TA98) colonies on the reversion-assay plate. The colonies seen on the sterility controls seem unlikely to be due to capricious contamination of some plates, given the large number of normal (0) sterility controls seen throughout the study. Note that values for sterility controls are reported in the tables only if they are *not* zero.

One explanation for these observations might be that contaminants present in the sample require some factor present on the sterility-control plate (e.g., a component of the S-9 protein extract) that is effectively removed by the inoculum of *Salmonella* on the reversion-assay plates, thus preventing growth of the contaminants. A second explanation might be that *Salmonella* simply divide faster than contaminants so that the nutrients on the reversion-assay plates are exhausted by the *Salmonella* before contaminating clones have grown to a visible size. At present these are the most reasonable hypotheses that we have found. Future study will be directed at the resolution of this paradox.

CONCLUSIONS

The assay of Illinois surface waters by the parfait/distillation method has detected significant mutagenicity in the Fox River at Aurora, Illinois, and the Salt Fork Creek at Urbana, Illinois. These results are interesting but must be interpreted cautiously considering the limited validations of the method and some unresolved inconsistencies in the results of the sterility controls in this study.

Table 18. Summary of Samples Taken for Mutagenicity Assay

Location	Date
1. Illinois River at Peoria; 200' upstream of sewage-treatment plant discharge	8/18/78
2. Peoria sewage plant secondary effluent, at point of discharge	8/18/78
3. Lake Decatur, at intake for south Decatur drinking-water treatment plant	8/31/78
4. Lake Bloomington, at intake for Bloomington drinking-water treatment plant	8/25/78
5. North Fork of Vermilion River at Danville water-treatment plant	9/14/78
6. Salt Fork Creek at Urbana, Perkins Rd. bridge — 6 h composite sample	9/19/78
7. Embarras River near Lawrenceville, IL, at intersection of Rt. 50 and Rt. 1	9/26/78
8. Fox River at Aurora, IL	9/26/78

Table A. Mutagenicity Assay of Water from Illinois River at Peoria, IL

Revertants of:		TA98			TA100	
Fraction	Vol extract: 0	1/8	1/80	0	1/8	1/80
A	82	63	72	111	128	96
B		203	88		438	150
C		0+C	77		0+C	100
D		84	87		206	151
E		89	78		122	104
F		106	90		164	130
G		87	78		2+C	90

Positive controls: TA98 with S-9 mix and 2 µg B(a)P=564
TA100 with 10 ng AF-2, without S-9 mix=464

Sterility controls: 1/80 vol of B=81, C=1, D=1, F=1, G=1

Toxic fractions: C,G

Mutagenic fractions: D? (see text)

Fraction B resterilized by filtration through 0.2µ Nuclepore filter and assay repeated: TA98 with S-9 mix, 1/8 vol=32
TA100 with S-9 mix, 1/12 vol=568
sterility, 1/80 vol=55
positive controls and background reversion frequency (see Table C.2)

Table B. Mutagenicity Assay of Secondary Effluent from Peoria Sewage-Treatment Plant

Revertants of:		TA98			TA100	
Fraction	Vol extract: 0	1/8	1/80	0	1/8	1/80
A	82	88	88	111	171	144
B		88	93		151	119
C		3+C	88		0+C	111
D		88	103		116	107
E		105	75		129	116
F		82	71		144	131
G		76	78		180	110

Positive controls: see Table A

Sterility controls: 1/80 vol of A-1, C=3, E=1

Toxic fractions: C

Mutagenic fractions: none

See also Tables C.1, C.2

Table C.1. Mutagenicity of Secondary Effluent from Peoria Sewage-Treatment Plant, Stored 29 d at 4° before Fractionation

Revertants of:		TA98			TA100	
Fraction	Vol extract: 0	1/8	1/80	0	1/8	1/80
A	49	41	35	104	104	128
B		37	40		174	121
C		Contam.	45		61+C	112
D		Contam.	53+C		159+C	144
E		29	39		120	106
F		33	36		128	113
G		44	34		118	111

Positive controls: TA98 with S-9 mix and 2 μg B(a)P=69
TA100 with S-9 mix and 10 ng 4-NQO=119

Toxic fractions: C

Mutagenic fractions: none, results inconclusive due to positive controls

Table C.2. Mutagenicity of Secondary Effluent from Peoria Sewage-Treatment Plant, Stored 29 d at 4° before Fractionation, Repeat Assay

Revertants of:		TA98		TA100	
Fraction	Vol extract: 0	1/8[a]	0	1/8[a]	
A	30	51	223	190	
B		53		200	
C		47		197	
D		Contam.		176+C	
E		42		190	
F		40		130	
G		46		172	

Positive controls: TA98 with S-9 mix and 2 μg B(a)P=109
TA100 with 20 ng 4-NQO, without S-9 mix=633

Toxic fractions: none

Mutagenic fractions: none

Inconclusive results: D

[a]Values of revertants from single plates only

Table D. Mutagenicity Assay of Duplicate, Parallel Samples of Water from Lake Decatur, IL

Revertants of:		TA98			TA100	
Fraction	Vol extract: 0	1/8	1/80	0	1/8	1/80
A	52	67+C	62	110	122+C	123
B		68	82		155	129
C		2+C	40+C		90+C	100
D		54+C	52+C		120	112
E		54+C	50+C		118	117
F		49	58		96	114
G[a]		ND	ND		ND	ND
H		53	45+C		127	98
I		78	50+C		131	112
J		47+C	46+C		75	124
K		Cont.	42+C		Cont.	96
L		48	52		106	118
M		60	109+C		130	132
N		33+C	44+C		ND[b]	181

Positive controls: TA98 with S-9 mix and 2 µg B(a)P=463
TA100 with 10 ng 4-NQO, without S-9 mix=286

Sterility controls: 1/80 vol of A=1, B=1, E=1, H=1, J=90, K=3, L=2

Toxic fractions: C,J,N

Mutagenic fractions: none

Inconclusive results: G,K

[a] Sample was lost.

[b] Results were obscured by loose semi-solid top agar. Repeat assay TA100 with S-9 mix=168. Repeat control, see Table C.2.

Table E. Mutagenicity Assay of Water from Lake Bloomington, IL

Revertants of:		TA98			TA100			TA1537	
Fraction	Vol extract: 0	1/8	1/80	0	1/8	1/80	0	1/8	1/80
A	40	50	51	125	80	85	33	44	29
B		45	38		88	92		43	32
C		2	46		2	112		0	19
D		75	43		107	120		38	42
E		42	40		88	100		38	36
F		33	56		86	92		33	34
G		30	33		78	79		25	38

Positive controls: TA98 with S-9 mix and 2 µg B(a)P=467
TA100 with 10 ng 4-NQO, without S-9 mix=896
TA1537 with S-9 mix and 100 µg 9-aminoacridine=58

Sterility controls: 1/80 vol of A=3, B=4, E=4, F=10, G=18

Toxic fractions: C

Mutagenic fractions: D?

Table F. Mutagenicity Assay of Water from North Fork of Vermilion River at Danville, IL

Revertants of:		TA98			TA100	
Fraction	Vol extract: 0	1/8	1/80	0	1/8	1/80
A	40	32	49	125	74	100
B		41	44		82	86
C		29+C	43+C		82	84
D		32+C	50		93	89
E		36	39		79	91
F		40+C	40		84	91
G		34	30		84	78

Positive controls: see Table E

Sterility controls: 1/80 vol of A=125, B=49, C=48, D=4, E=98, F=78, G=117

Toxic fractions: none

Mutagenic fractions: none

Table G.1. Mutagenicity Assay of Composite Sample of Water from Salt Fork Creek, Urbana, IL

Revertants of:		TA98			TA100	
Fraction	Vol extract: 0	1/8	1/80	0	1/8	1/80
A	>2000			104	117	103
B					117	117
C					119+C	110
D					135	115
E		All assay plates contam.			95	119
F					98	107
G					158	110

Positive controls: TA98 with S-9 mix and 2 µg B(a)P > 2000
TA100 with 10 ng 4-NQO, without S-9 mix=119

Sterility controls: 1/80 vol of A=8, C=9

Toxic fractions: none

Mutagenic fractions: none; results inconclusive due to positive controls and contamination

Table G.2. Mutagenicity Assay of Composite Sample of Water from Salt Fork Creek, Urbana, IL, Repeat Assay

Revertants of:		TA98[a]		TA100[a]	
Fraction	Vol extract: 0	1/8	0	1/8
A	30	54	223	167
B		42		182
C		24+C		261
D		46+C		168+C
E		49		191
F		46		308
G		46		551

Positive controls: see Table C.2

Sterility controls: not repeated, see Table G.1

Toxic fractions: none

Mutagenic fractions: G

[a] Values of revertants from single plates only

Table H.1. Mutagenicity Assay of Water from Embarras River at Lawrenceville, IL

Revertants of:		TA98			TA100	
Fraction	Vol extract: 0	1/8	1/80	0	1/8	1/80
A	58	62	65	156	164	182
B		32	54		183	133
C		22+C	44+C		26	138
D		78	56		176	150
E		56	55		152	144
F		66	69		134	156
G		46	48		202	127

Positive controls: TA98 with S-9 mix and 2 µg B(a)P-4,5-oxide=95
TA100 with S-9 mix and 10 ng 4-NQO=172

Sterility controls: 1/80 vol of C=371, D=49, E=6, F=3, G=10

Toxic fractions: C

Mutagenic fractions: none, results inconclusive due to positive controls and sterility controls

Table H.2. Mutagenicity Assay of Water from Embarras River at Lawrenceville, IL, Repeat Assay

Revertants of:		TA98			TA100	
Fraction	Vol extract: 0	1/8[a]	1/80	0	1/8[a]	1/80
A	22	19+C	21	106	106	140
B		0	17		29	67
C		15	23		114	89
D		40	22		109	88
E		35	18		142	93
F		25	16		86	90
G		17	17		130	102

Positive controls: TA98 with S-9 mix and 2 µg B(a)P=334
TA100 with 10 ng 4-NQO, without S-9 mix=641

Sterility controls: 1/80 vol of A=1, B=14, C=2, G=2

Toxic fractions: B

Mutagenic fractions: D?

[a] Values of revertants from single plates only

Table I.1. Mutagenicity Assay of Water from Fox River at Aurora, IL

Revertants of:		TA98			TA100	
Fraction	Vol extract: 0	1/8	1/80	0	1/8	1/80
A	58	66	70	156	234	150
B		0	54		0	151
C		Contam.	Contam.		Contam.	Contam.
D		54	64		117	176
E		60	59		144	169
F		58	60		130	152
G		62	54		520	168

Positive controls: see Table H.1

Sterility controls: 1/80 vol of A=6, B=6, C=152, D=11, E=6, F=123, G=912

Toxic Fractions: B

Mutagenic Fractions: G, results inconclusive due to positive controls and sterility controls

Table I.2. Mutagenicity Assay of Water from Fox River at Aurora, IL, Repeat Assay

Revertants of:		TA98			TA100	
Fraction	Vol extract: 0	1/8[a]	1/80	0	1/8[a]	1/80
A	22	Contam.	3816	106	Contam.	2884
B		0	21		0	116
C		Contam.	216		Contam.	796+C
D		21	32		94	127
E		24	21		108	118
F		15	19		83	102
G		18+C	14		427	116

Positive controls: see Table H.2

Sterility controls: 1/80 vol of A=11, B=14, C=26, E=2, G=3

Toxic fractions: B

Mutagenic fractions: G, C?

Inconclusive fractions: A , C

[a] Values of revertants from single plates only

Table J. Summary of Samples Showing Toxicity or Mutagenic Activity

Sample		Toxic Fractions	Mutagenic Fractions
Illinois River	Table A	C,G	D?
Peoria Effluent	Table B	C	--
Lake Decatur	Table D	C,J,N	--
Lake Bloomington	Table E	C	D?
Salt Fork Creek	Table G.2	--	G
Embarras River	Table H.2	B	D?
Fox River	Table I.2	B	G, C?

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