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Use of Ames Test in Evaluation of Shale Oil Fractions

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Conditions that affect the sensitivity of the Ames assay of complex hydrocarbon mixtures derived from shale oil were studied. Two fractions, one enriched in polynuclear aromatic compounds (PNA fraction), and a second fraction enriched in aromatic and heterocyclic amines (basic fraction), were selected for most of this work because of their comparatively high mutagenicity (i.e., compared with raw shale oil). The crude shale oil, as well as the basic, PNA, and tar fractions were mutagenic against the *Salmonella typhimurium* test strains, TA98 and TA100. Mutation was dependent on metabolic activation by microsomal (S9) enzymes. Both test strains responded equally well to the crude product and to the basic fraction; however, strain TA100 was more effective than TA98 in demonstrating the mutagenicity of the PNA fraction. The mutagenicity of the tar fraction could be most easily detected after metabolic activation in a liquid medium, as opposed to S9 activation in the top agar of the standard Ames assay. The mutagenicity of the basic fraction or 2-aminoanthracene was also demonstrated by metabolic activation in a liquid medium. In another set of experiments, the effect of chemical composition on the expression of mutagenicity in the standard Ames assay was estimated. Premutagens requiring metabolic activation were added to the basic and PNA fractions, and the numbers of revertants obtained in the presence of the fractions were compared with mutation induced by the compounds alone. The basic fraction did not interfere with the mutagenicity of 2-aminoanthracene and 7,9 dimethylbenz[c]acridine. Moreover, in certain experiments, the mutagenicity of the complex fraction plus the added compound was higher than expected on the basis of assays performed on these materials separately. Conversely, the PNA fraction prevented or strongly inhibited mutation by several polynuclear aromatic compounds, and an acridine. However, the PNA fraction did not inhibit mutation induced by 2-aminoanthracene. The effect of the basic fraction on stability of the S9 enzymes in the standard Ames test was also determined.

Introduction

Raw shale oil is a complex chemical, differing from most crude petroleums in having comparatively high concentrations of basic (nitrogen-containing) and phenolic compounds (*1*). However, shale oil also contains aliphatic, olefinic, and aromatic constituents which are more commonly associated with crude oil. Complex hydrocarbon mixtures such as shale oil may pose special problems when used for prescreening of mutagenic materials with the Ames assay. Hydrocarbon mixtures are more likely to be cytotoxic to test organisms than pure compounds at the concentrations required for mutagenesis, reducing the sensitivity of the Ames test. In addition to problems associated with cellular toxicity, complex chemical mixtures may decrease the sensitivity of the Ames test by interfering with the microsomal (S9) enzyme reactions which form active mutagens from premutagenic precursors, i.e., metabolic activation reactions. In this regard, both inhibition and inactivation of S9 enzymes are possibilities that must be considered. In addition, inhibition of mutation in the Ames assay may also arise even when S9 enzymes are fully active. These enzymes in many instances lack substrate specificity (*2–4*). Such lack of specificity is advantageous for demonstrating the mutagenicity of many structurally different compounds. On the other hand, lack of enzyme specificity increases the possibility for nonmutagen-producing reactions when complex chemical mixtures are assayed. For example, simple aromatics and aliphatic compounds can be attacked by the mixed function oxidases present in microsomal S9 enzymes (*5*). However, these reaction products may not be mutagenic in the Ames test.

In the work described here we investigated the effect of chemical composition (rather than simply

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the concentration of individual compounds) on the sensitivity of the Ames assay of a shale oil. To do this, a sample of a raw shale oil and several of its principal chemical subfractions were tested for mutagenicity against two of the *Salmonella typhimurium* histidine auxotrophs of Ames et al. (*6*). Both the standard Ames assay and a simple modification of this procedure were used to demonstrate the mutagenicity of these complex materials.

The standard assay was carried out by mixing of all components, including the complex fraction, test cells, and cofactors into a semisolid agar layer which was spread onto selective agar medium. Mutagenized cells thus gave rise to revertant (histidine-independent) colonies which were scored in the assay. In the modified procedure, complex fractions were assayed by first exposing cells to them in a liquid medium containing the S9 enzymes. After the activation-exposure step, small aliquots were removed and diluted into top agar for spreading onto the same selective medium as in the Ames assay. In this second procedure, the net effect was thus a reduction in the concentration of mutagen or complex fraction contained in the top agar of the selective medium used in the Ames test. Moreover, the modified assay permits control of the time during which cells are exposed to complex fractions.

Finally, the effect of complex fractions on the sensitivity of the standard Ames test was estimated. Standard premutagenic chemicals were added to complex fractions and the mutagenicity of the resulting mixture was compared with the mutagenicities of compounds and fractions assayed separately. In related experiments, we also tried to determine the stability of S9 enzymes in the presence of complex fractions.

Methods

Bioassays

Salmonella typhimurium TA98 and *S. typhimurium* TA100, two of the strains developed by Ames et al. (*6*), were used in this work. The standard Ames test has been described in detail (*7*). The modified Ames test was carried out as follows: The shale oil fraction to be tested was added to a test tube containing microsomal (S9) enzymes and also to a control tube which contained buffer but no S9 enzymes. The total volume for the preincubation step was 0.5 ml. After a 30-min incubation period, 1 ml of an overnight culture of *S. typhimurium* in nutrient broth was added to the test tubes containing the oil fractions (± S9 enzymes), and incubation was continued for an additional 30 min. After the 30-min exposure, 150 μl (ca. 10^8 cells) were removed and added to semisolid top agar (4 ml) containing the components of the standard Ames assay. The molten top agar was then poured onto selective medium to allow the expression of mutagenicity in terms of revertant colony formation. Incubation of these assay agar Petri plates was done at 37°C. Mutant colonies were counted after 24–36 hr. Subsequent to addition of cells to the molten top agar, the modified assay was the same as the standard assay. The purpose of the modified assay was to expose cells to relatively high levels of shale oil fraction in such a way that the final concentrations of the fraction could be rapidly reduced by dilution to a point where toxicity or killing of cells did not seriously affect the assay.

Estimation of Cell Numbers

Numbers of viable *S. typhimurium* cells were estimated by direct dilution plate counts of growing cultures versus optical density (540 mm). In order to measure the dependence of mutation on cell number, an overnight culture of *S. typhimurium* TA98 or TA100 was diluted with 0.05*M* phosphate buffer, pH 7.0, until a linear response was obtained for concentration versus optical density. The number of cells was then estimated from the standard curves relating optical density to viable cells (i.e., colony-forming units).

Kinetics of Microsomal Enzyme Inactivation

In order to estimate the stability of the microsomal enzymes in the standard Ames test system, the following experiment was carried out. The crude S9 microsomal enzymes plus the substrates required for metabolic activation [nicotinamide adenine dinucleotide phosphate (NADP), a glucose-6-phosphate], and a chemical known to require metabolic activation (e.g., 2-aminoanthracene, benzopyrene) was added to 4 ml of liquid top agar held at 45°C. This molten top agar solution was then poured onto a glucose mineral base agar plate and allowed to solidify as in the standard Ames assay (*7*). The solidified top agar layer thus contained the microsomal or S9 enzyme substrates for generating active metabolite-mutagens from the inactive polyaromatic precursors and the polycyclic compound or complex fraction being assayed; however, no *S. typhimurium* test cells were added to the top agar during this first step. Then, at various time intervals after the mutagen-generating system was initiated, 4 ml of a second top agar layer containing approximately 2.0×10^8 cells of a *S. typhimurium* test strain, was poured onto the first layer. After 24–36 hr of incubation at

37°C, the agar plates (containing both top agar layers) were counted for the number of revertant colony-forming units. The mutational response (revertant colonies per plate) was thus measured as a function of preincubation time after initiation of the S9 reactions.

Preparation of Chemical Mutagen-Carcinogen Solutions

The following mutagen carcinogens were used in our experiments: 2-aminoanthracene; benzo[a]pyrene (benzopyrene), benz[a]anthracene (benzanthracene), 7,9-dimethylbenz[c]acridine (dimethylbenzacridine). Stock solutions of these compounds ranging from 1000 to 2000 μg/ml were prepared in dimethylsulfoxide (DMSO).

Preparation of Microsomal Enzymes

The induction procedure followed the general method of Czygan et al. (*8*). The preparation of the microsomal (S9) liver enzymes was done according to the method of Garner et al. (*9*). At time of sacrifice, livers of the rats were immediately perfused with 30 ml of ice-cold 0.154*M* KCl before excision and kept ice cold through each of the subsequent preparative steps. After centrifugation at 9000*g*, liver homogenates were quickly frozen in a Dry Ice bath and stored at −70°C until use. Protein concentration of the S9 rat liver homogenates was estimated by the method of Lowry et al. (*10*).

Preparation of Shale Oil Fractions

A sample of a raw shale oil was obtained from the small retort (125 kg) at the Lawrence Livermore Laboratory. It was generated by simulated, modified *in situ* pyrolysis of Anvil Points oil shale in run S-11 (designated as shale oil S-11 in this paper). It is a research material for use in physical-chemical and biological experimentation, and cannot be considered as a representative sample from a pilot plant or from a commercially oriented process. The scheme outlined in Figure 1 was used to separate crude shale oil into five complex fractions. To begin this extraction procedure, about 5 ml of shale oil was shaken with 100 ml of isooctane and allowed to stand several hours for the insoluble tar residue to settle. The isooctane solution was decanted, then treated with aqueous 1*N* HCl (3 × 50 ml). The aqueous extracts were combined, their pH adjusted to 11, and they were then re-extracted with isooctane (3 × 5 ml). The isooctane extracts were dried over anhydrous sodium sulfate and concentrated on a rotary evaporator to recover the basic compo-

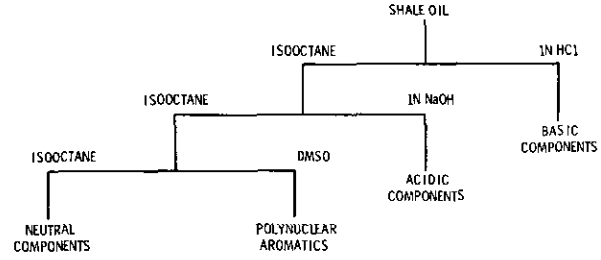

FIGURE 1. Fractionation scheme for shale oil S-11. See text for details.

nents. The isooctane solution of the dissolved oil (minus basic components) was next extracted with aqueous 1*N* NaOH (3 × 50 ml). The alkaline aqueous extracts were combined, acidified to pH 3 with HCl, then extracted as described above to recover acidic components. The remaining organic phase was then extracted with DMSO (3 × 50 ml). The DMSO extracts were combined, diluted with 150 ml of distilled water, and extracted with isooctane to recover the polynuclear aromatic (PNA) hydrocarbons. The remaining organic phase, containing non-PNA neutral components, was dried and concentrated as described above. The concentrated acidic, basic, neutral, PNA, and tar fractions derived by this procedure were flashed down under reduced pressure (~ 1 mm Hg) to remove as much isooctane as possible. The qualitative composition of the five complex fractions was studied by using gas chromatography/mass spectrometry (GCMS). The neutral fraction contains mostly *n*-alkanes, 1-alkenes, along with some cycloalkanes and the low molecular weight aromatic compounds, benzene, toluene, etc. The acid fraction contains phenolic compounds and a large number of as-yet unidentified compounds. The PNA fraction contains compounds ranging from naphthalene (molecular weight 128) up to at least the benzopyrenes (molecular weight 252). The basic fraction is primarily alkyl-substituted pyridines and quinolines. However, various benzoquinolines have also been detected. Larger compounds, with molecular weights corresponding to naphthylamines (molecular weight 143) and (molecular weight 195), are also present. For use in the bioassays, these fractions, or the corresponding unfractionated raw shale oil, were solubilized in DMSO for direct addition to either the standard or modified Ames test system.

Results

Mutagenicity of a Shale Oil

A crude shale oil was separated into five fractions, representing classes of chemically different

compounds: acidic, basic, neutral, polynuclear aromatic (PNA), and a residual or tar fraction (see Methods). Each of the chemically complex fractions was then assayed for mutagenicity against *S. typhimurium* strains TA100 or TA98.

The data shown in Figures 2 and 3 were obtained by adding the microsomal S9 enzymes directly to the top agar layer along with the *Salmonella* test strain and the crude fraction. In this technique, the cells are continuously exposed to the shale oil fraction and to mutagens formed by the S9 enzymes for the length of time that the microsomes are active. As described in the Methods Section, this situation will be referred to here as the standard Ames test or assay.

The basic and PNA fractions, and the crude shale oil, were mutagenic against TA98 (Fig. 2), with the basic fraction the most active (1 revertant colony/μg). Lower rates were observed for the crude shale oil and the PNA fractions. The acidic, neutral, and tar fractions showed little or no mutagenicity except at the highest concentration of the tar fraction tested.

In a comparable experiment, the shale oil and the complex fraction were tested against strain TA100 (Fig. 3). The response curves for the basic fraction and crude shale oil were similar to those for TA98. However, the PNA fraction and the residual tar fraction were more mutagenic than for strain TA98. The mutagenicity of shale oil S-11 and its subfractions was strictly dependent on the presence of S9 enzymes in the assay system, regardless of the *S. typhimurium* strain used in the test (data not shown).

An alternate method of mutagenizing cells is illustrated by the data shown in Figures 4-6. In this method, S9 enzymes, plus either crude shale oil, complex fraction, or pure chemicals, were added to a liquid medium in a pre-incubation step (Methods). This was followed by addition of the *Salmonella* test strain (TA98) in the exposure step and dilution of the exposed cells into the molten top agar for plating as in the standard Ames assay. This second method of mutagenizing cells will be referred to as the modified assay. It should be noted that the mutagenicity for each fraction or chemical mutagen was demonstrated by both of the assay techniques. The arylamine, 2-aminoanthracene, was metabolically activated in both the modified and standard Ames assay systems (Fig. 4). Based on the initial or starting concentration of 2-aminoanthracene (1/10 dilution of the pre-incubation mix), mutational response per assay plate was approximately the same for both tests. However, if the comparison was made by using the total initial concentration of 2-aminoanthracene in the liquid exposure-activation

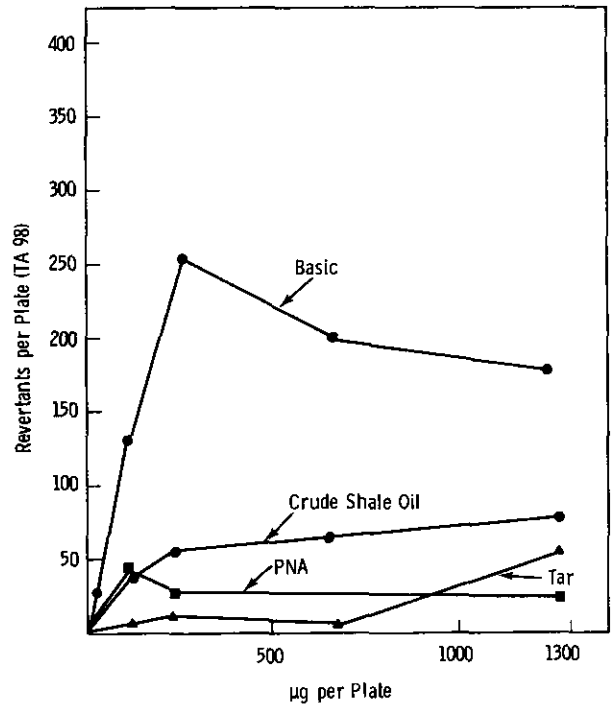

FIGURE 2. Mutagenicity of shale oil S-11 against *Salmonella typhimurium* TA98. Spontaneous revertants of about 20 per plate have been subtracted. Each plate contained 1500 μg S9 protein.

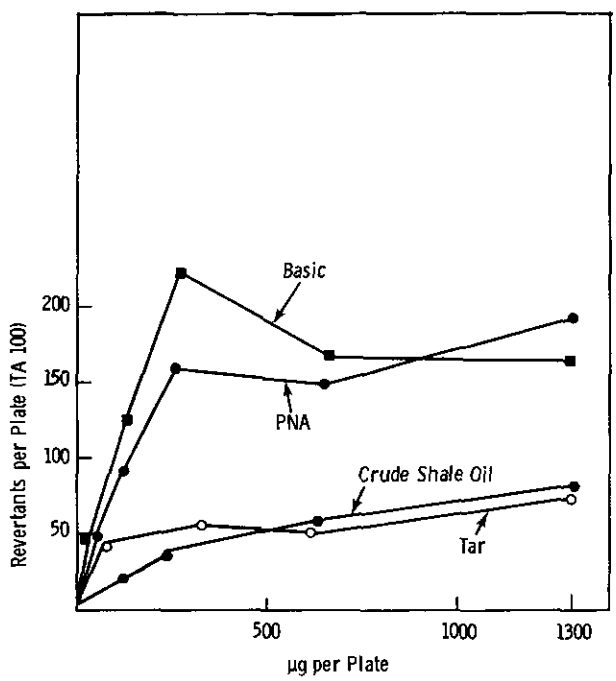

FIGURE 3. Mutagenicity of shale oil S-11 against *Salmonella typhimurium* TA100. Spontaneous revertants of about 70 per plate have been subtracted. The concentration of S9 protein was 1500 μg per plate.

step, the mutational response for the modified assay was considerably lower than observed for the standard test.

The basic fraction was also mutagenic in the modified assay (Fig. 5). In this case, however, the number of revertant colonies formed after exposure of cells to basic fraction in liquid medium was approximately equal to the number obtained for the standard assay, on the basis of response per μg complex material initially present. On the other hand, the mutational response after activation in liquid was greater than that observed in the standard assay, using initial concentration of basic fraction as the basis for comparison, and taking into account that only 1/10 of this material could have been transferred to the selective medium used in the assay step. The tar fraction (Fig. 6) was slightly mutagenic in the standard assay, but showed a comparatively strong response in the modified assay. These data suggest that the modified assay is preferable for determining the tar fraction of shale oil S-11.

Mutagenicity in the Presence of Crude Fractions

A potential problem in applying the standard Ames test to complex chemical mixtures is the possibility that a loss of sensitivity will occur due to composition, i.e., that the mutagenicity of a complex material may be less than the sum of those of the individual components.

One method of estimating the importance of the composition of complex mixtures on the Ames assay is to add a chemical mutagen to a complex fraction, then compare the mutagenicity of the mixed system (chemical + fraction) with the mutagenicity of the chemical alone. This experimental approach was used with the basic and PNA fractions as representative complex materials, and 2-aminoanthracene, benzanthracene, benzopyrene, and dimethylbenzacridine as the known chemical mutagens (i.e., premutagens). The parameter of interest in each of these experiments is the ratio of weight complex fraction to weight of chemical. This value was controlled in two ways. First, the concentration of the chemical was held at a constant level and different amounts of complex fraction were added to the system. In the second method, the concentration of the complex fraction was held constant and the chemical was varied.

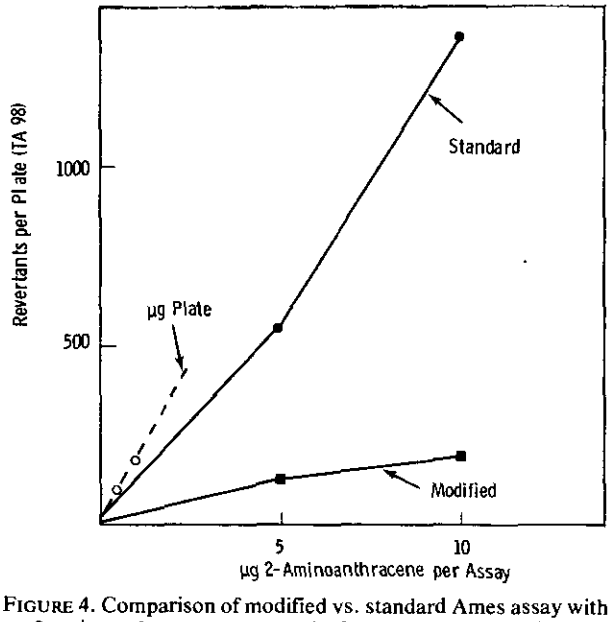

FIGURE 4. Comparison of modified vs. standard Ames assay with 2-aminoanthracene: (●) standard assay; (■) modified assay; (○) calculated response for modified assay based on initial concentration of 2-aminoanthracene carried over to the assay plate. Each sample contained 500 μg of S9 protein in the top agar or in the liquid pre-incubation mix. The background number of spontaneous revertant colonies was subtracted from the total.

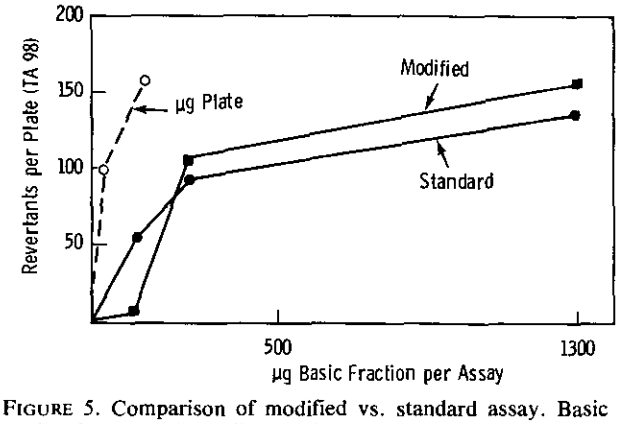

FIGURE 5. Comparison of modified vs. standard assay. Basic fraction from shale oil S-11. Conditions and symbols are the same as for Fig. 4.

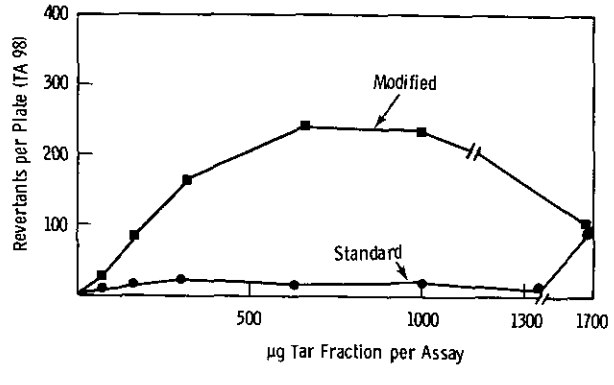

FIGURE 6. Comparison of modified vs. standard assay. Tar fraction from shale oil S-11. Conditions and symbols are the same as for Fig. 4.

From the first method, it can be seen that the mutagenicity of 25 μg benzanthracene in the standard Ames assay decreased sharply with increasing concentrations of the PNA fraction (Fig. 7). At a ratio of 10:1 PNA fraction to benzanthracene, the number of revertant colonies per plate fell by approximately 50%. Increasing this ratio to 50:1 resulted in an approximate 94% decrease in the number of revertant colonies formed. By the second method, the concentration of benzanthracene was varied against a fixed concentration of the PNA fraction (Fig. 8). As can be seen, revertant colonies were formed at the rate of approximately 45 colonies/μg benzanthracene in the linear portion of the response curve. However, in the presence of the PNA fraction, little, if any increase in the number of revertant colonies was observed over the value obtained for the PNA fraction alone. Similar results were also obtained for benzopyrene, which is structurally similar to benzanthracene (Fig. 9). In the linear portion of the response curve, approximately 175 colonies were formed/μg of benzopyrene added to the Ames test system. With a fixed level of the PNA fraction present, the rate of revertant colony formation/μg benzopyrene decreased more than 80%, i.e., from 175 colonies/μg

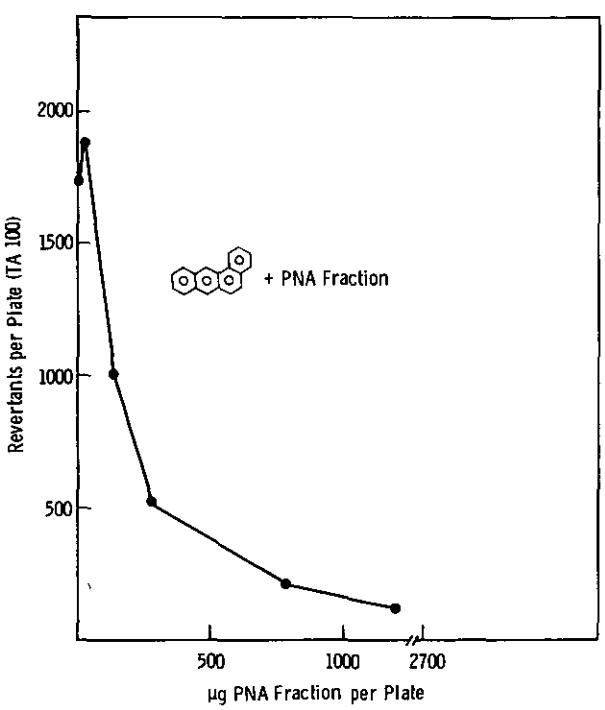

FIGURE 7. Effect of PNA fraction on mutagenesis by benz[a]anthracene. Concentration of benz[a]anthracene was fixed at 25 μg/plate. Spontaneous revertant colonies estimated at 90 per plate were subtracted for the figure and for Figs. 8-12. Each plate contained approximately 2000 μg of S9 protein.

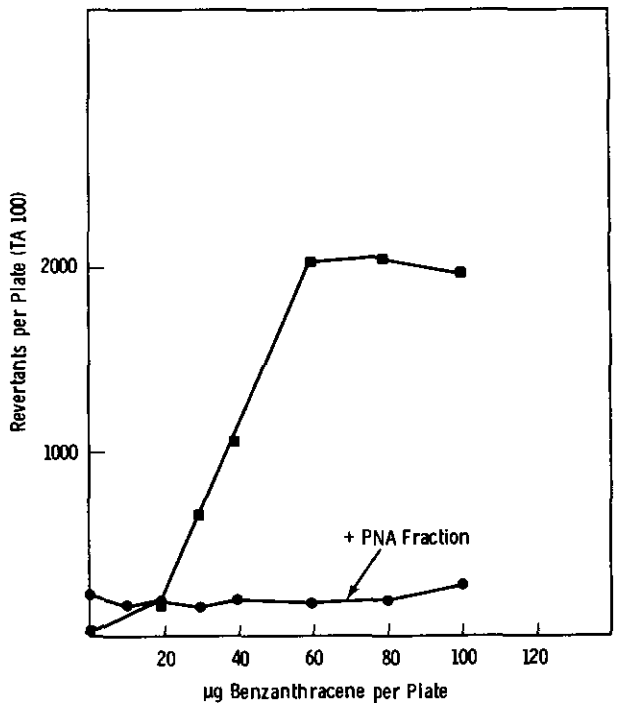

FIGURE 8. Effect of PNA fraction on mutagenesis by benz[a]anthracene; concentration of PNA fraction fixed at 1377 μg/plate: (■) response curves for the chemical alone; (●) chemical plus PNA fraction. Each plate contained approximately 2000 μg S9 protein.

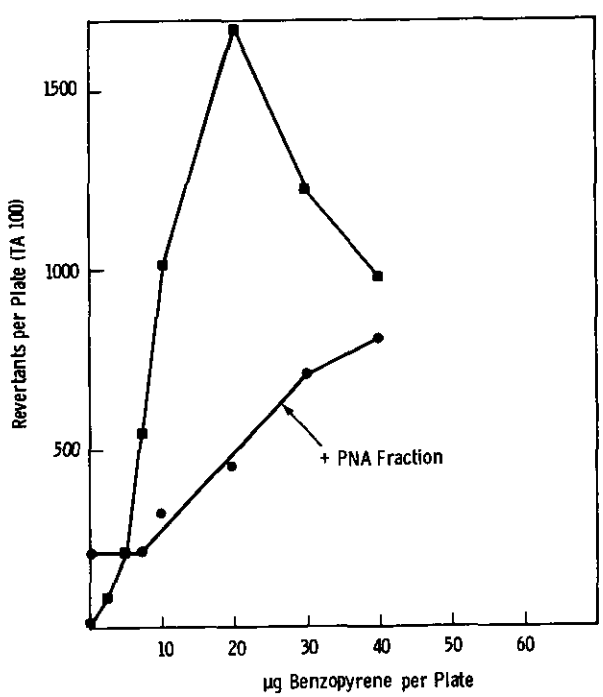

FIGURE 9. Effect of PNA fraction on mutagenesis by benzo[a]pyrene: (■) chemical alone; (●) chemical plus PNA fraction. Concentration of PNA per plate and the concentration of S9 enzymes were the same as for Fig. 8.

to less than 30 revertant colonies/μg. The effect of the PNA fraction on the mutagenicity of the nitrogen-containing compounds, 2-aminoanthracene and dimethylbenzacridine, was also determined. The mutagenicity of 25 μg of dimethylbenzacridine as a function of increasing PNA concentration (Fig. 10) was inhibited in much the same way as observed for benzopyrene and dibenzanthracene. On the other hand, the PNA fraction had very little effect on the mutagenicity of 2-aminoanthracene (Fig. 11).

In experiments comparable to those just described for the PNA fraction, the effect of the basic fraction on the mutagenicity of the nitrogen-containing premutagens 2-aminoanthracene and dimethylbenzacridine was determined. As can be seen (Fig. 12), the number of revertant colonies obtained from 10 μg of 2-aminoanthracene alone was reduced about 50% when the basic fraction was in excess at a ratio of 30:1 relative to the aromatic amine. At higher concentrations of basic fraction, there was little increase in the level of inhibition except at the highest concentration of basic fraction tested in this experiment. The response curves obtained for 2-aminoanthracene and dimethylbenzacridine were similar in the levels of maximum response in the presence or absence of the basic fraction (Figs. 13 and 14). However, the rates of mutagenesis were somewhat reduced by the basic fraction. For example, 2-aminoanthracene alone yielded approximately 280 revertants/μg chemical added to the assay system. With the basic fraction present, this rate decreased about 60% to a value of approximately 120 revertants/μg of added 2-aminoanthracene. The comparable values for the dimethylbenzacridine were 120 and 102 revertants/μg added chemical (i.e., 15% reduction), in the presence and absence, respectively, of the basic fraction. In these experiments, the basic fraction was moderately inhibitory to mutagenesis. However, in other experiments, mutagenesis by 2-aminoanthracene appeared to be enhanced relative to fraction or chemical alone.

Kinetics of Mutagen Formation

Metabolic activation in the standard Ames assay depends on both the stability and the activity of the microsomal S9 enzymes. One approach for estimating how these enzyme systems are affected by complex chemical fractions from shale oil is shown

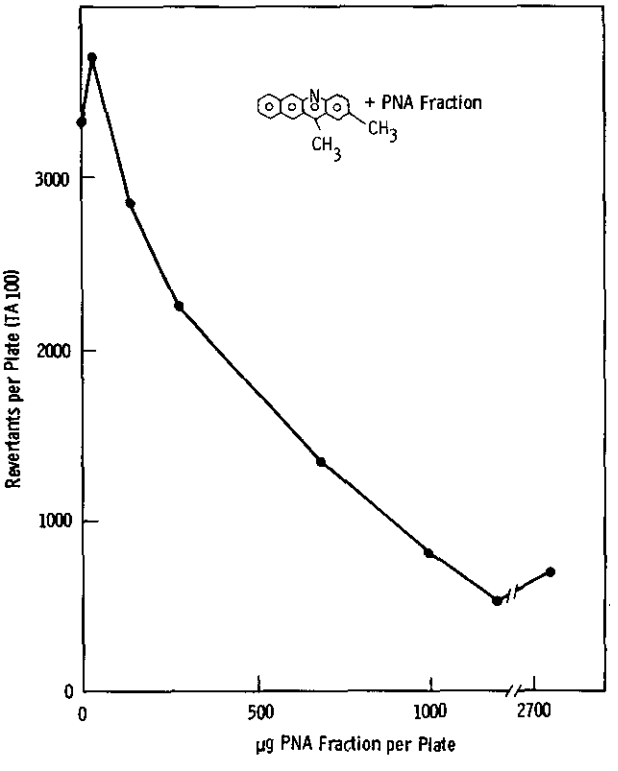

FIGURE 10. Effect of PNA fraction on mutagenesis by 7,9-dimethylbenz[c]acridine. Concentration of the chemical was fixed at 25 μg per plate.

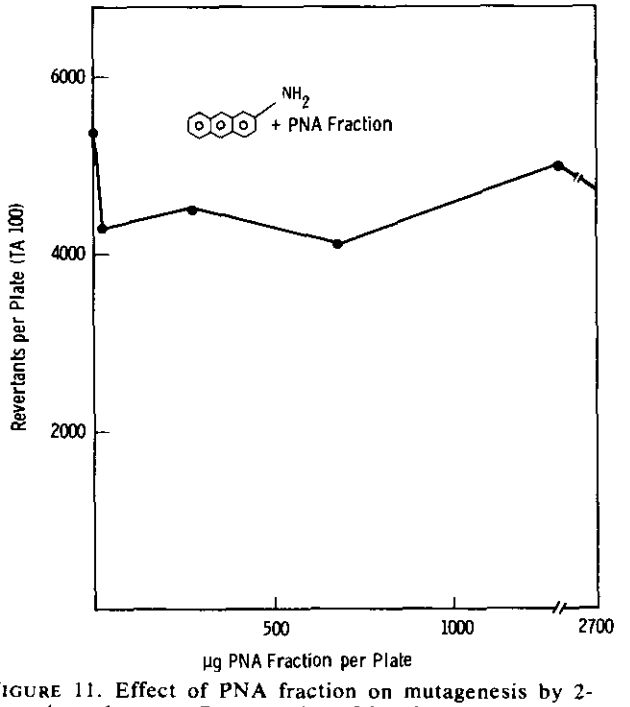

FIGURE 11. Effect of PNA fraction on mutagenesis by 2-aminoanthracene. Concentration of 2-aminoanthracene was fixed at 10 μg per plate. Concentration of S9 enzymes and the background of spontaneous revertants per plate was the same as for Fig. 7.

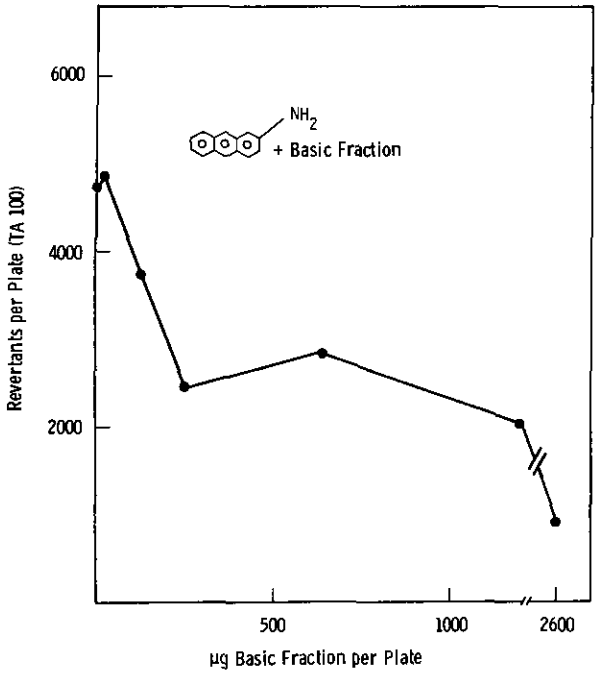

FIGURE 12. Effect of basic fraction on mutagenesis by 2-aminoanthracene. The concentration of 2-aminoanthracene was fixed at 10 μg per plate. Number of spontaneous revertant colonies and concentration of S9 enzymes per plate were the same as for Fig. 7.

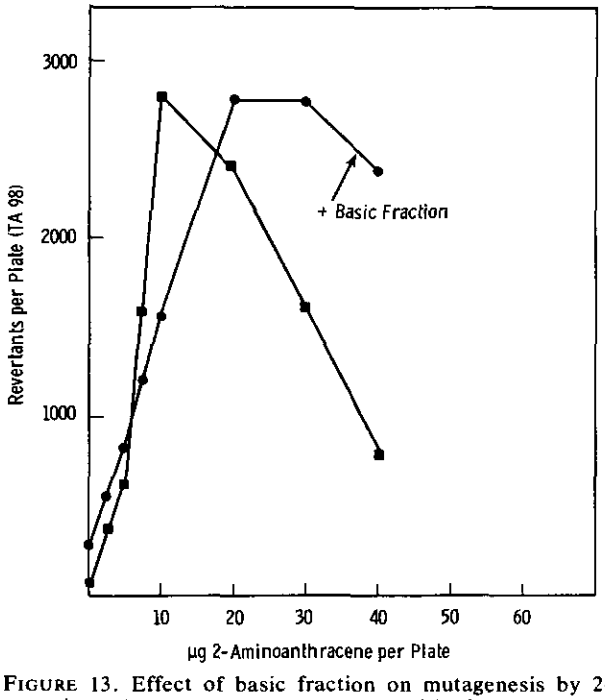

FIGURE 13. Effect of basic fraction on mutagenesis by 2-aminoanthracene: (●) concentration of basic fraction per plate was fixed at 1295 μg; (■) response curve for 2-aminoanthracene alone. Spontaneous revertant colonies estimated at 100 per plate were subtracted. Each plate contained approximately 2000 μg S9 protein.

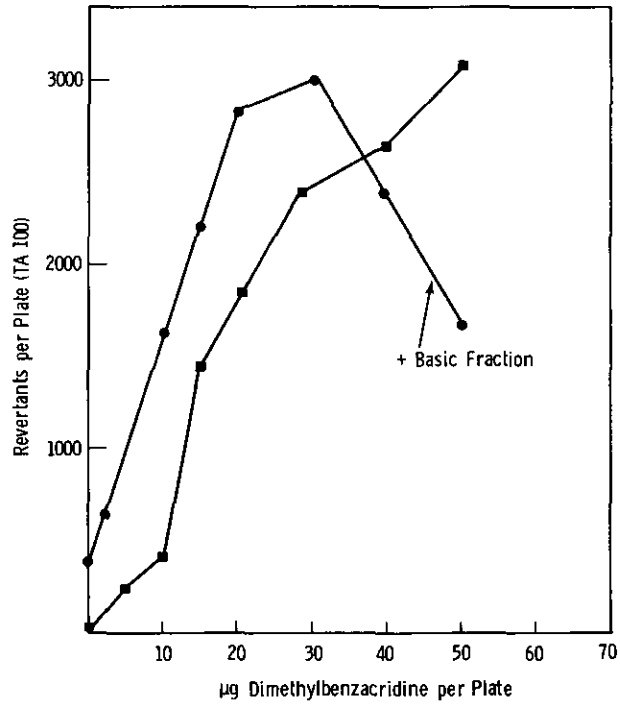

FIGURE 14. Effect of basic fraction on mutagenesis by dimethylbenz[c]acridine: (■) response curve for the chemical alone; (●) chemical plus the basic fraction. Conditions were the same as for Fig. 12.

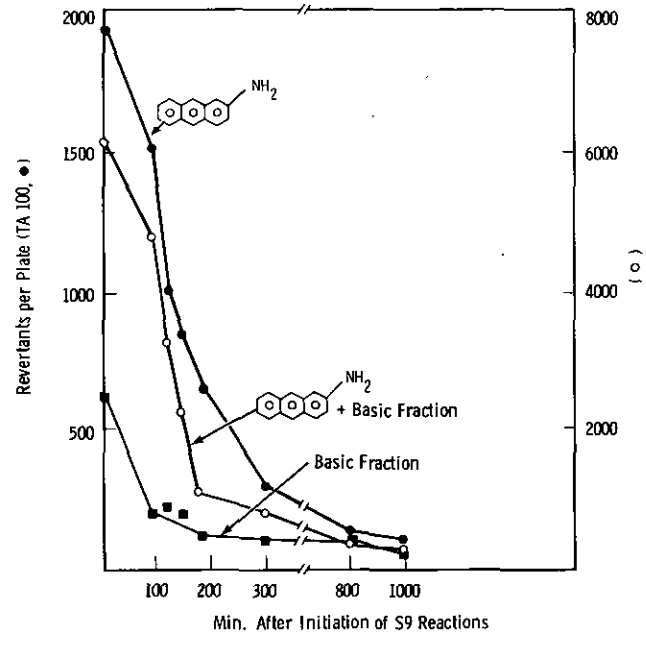

FIGURE 15. Kinetics of mutagen formation for 2-aminoanthracene (10 μg) with and without the basic fraction (1295 μg): (●) chemical alone; (■) basic fraction; (○) chemical plus basic fraction. S9-catalyzed reactions were initiated at 0 min and test organisms were added at times indicated. Concentration of S9 enzymes was approximately 2000 μg per plate.

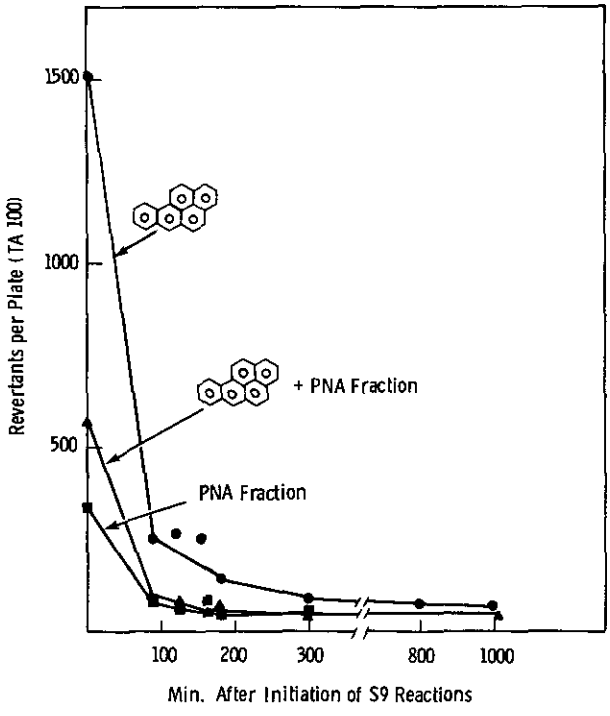

FIGURE 16. Kinetics of mutagen formation for benzo[a]pyrene (25 μg) with and without the PNA fraction (1377 μg): (●) chemical alone; (■) PNA fraction; (▲) chemical plus PNA fraction. Conditions were similar to those of Fig. 14.

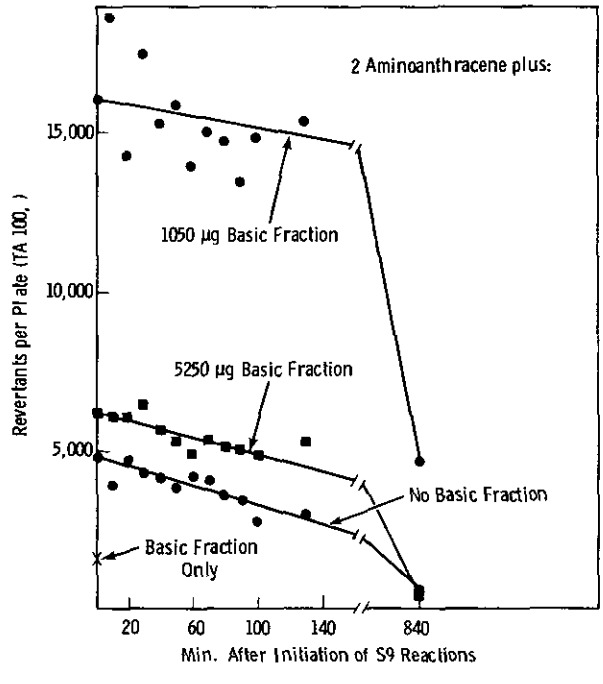

FIGURE 17. Kinetics of mutagen formation for 2-aminoanthracene (30 μg) with and without two concentrations of basic fraction. Conditions of the experiment are the same as for Fig. 14.

by the data in Figures 15-17. In these experiments, the complete mutagen-generating system, i.e., substrates, premutagens, and complex fractions, were mixed together in liquid semisolid agar, then added to the agar Petri plates used in the assay and allowed to harden (Methods). At various time intervals after initiation of this S9 reaction in the top agar layer, a second top agar layer, containing the *Salmonella* test strain TA100 was poured onto the first one, which contained the S9 enzymes. Mutagenesis in this system occurs by diffusion of metabolites across the boundary separating the two agar layers. Thus, the potential for mutagenesis was measured as a function of time after the initiation of the microsomal enzyme reactions.

The potential for mutagenesis, expressed by the number of revertant colonies formed after addition of TA100, decreased rapidly in the time interval from 0 to 300 min for 2-aminoanthracene (10 μg) and benzopyrene (25 μg). The corresponding decay rates were not greatly changed by the presence of either the basic fraction or the PNA fraction (Figs. 15 and 16). Increasing the concentration of 2-aminoanthracene extended the lifetime of the mutagen-generating system. Again, the presence of the basic fraction had little effect on the loss of mutagenicity potential. However, the number of revertant colonies formed by assay of the mixtures (chemical + fraction) vs. chemical alone gave evidence that chemical composition was significantly affecting the level of the mutational response. For the combination, 2-aminoanthracene plus basic fraction, the mutational response exceeded the sum of responses (revertants) for 2-aminoanthracene and basic assayed separately. This stimulation ranged up to threefold (Figs. 15 and 17). The maximum stimulation occurred at approximately 1 mg basic fraction per plate and fell off considerably at 5 mg basic fraction per plate. Conversely, the mutational response for benzopyrene plus PNA fraction was considerably lower than the sum of revertant colonies obtained for the chemical and the complex fraction alone. Under the experimental conditions used in this series of experiments the concentration of glucose-6-phosphate and NADP did not rate-limit the Ames assay (Fig. 18).

The components of the basic and PNA fractions were partially resolved by GCMS (Figs. 19 and 20). As can be seen, constituents of the basic fraction included alkyl-substituted anilines, pyridines, pyrroles, and quinolines. Compounds with molecular weights corresponding to either aminonaphthalenes or alkyl-substituted quinolines were also detected. In all, 31 of the peaks in Figure 18 were assigned identities (or probable indentities) based on comparison with standard reference compounds. In ad-

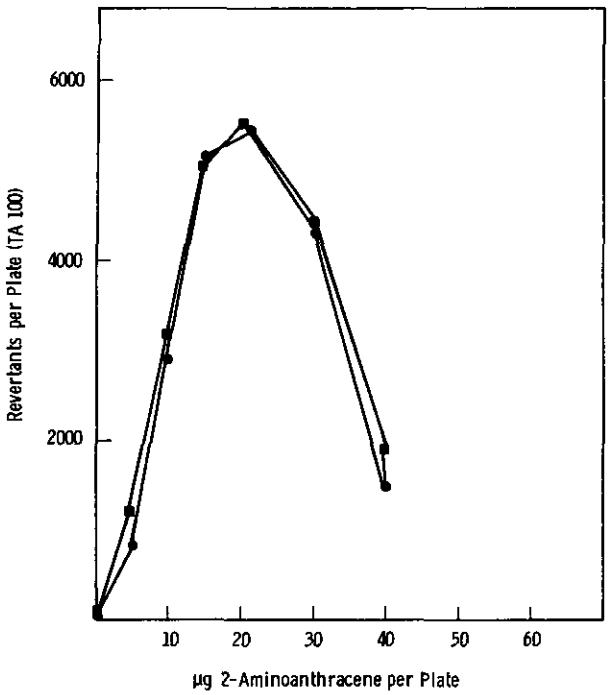

FIGURE 18. Effect of increasing concentrations of glucose-6-phosphate (5 to 20 μmole) and NADP (5 to 20 μmole) on standard Ames assay of 2-aminoanthracene: (●) concentrations used in the standard assay; (■) fourfold concentration increase. Concentration of S9 enzymes was approximately 2000 μg per plate.

dition, a large number of peaks remained unidentified.

The composition of the PNA fraction was also partially resolved by the GCMS separation procedure. Sixteen compounds, with molecular weight ranging from that of naphthalene to benzopyrene were identified. Compared with the basic fraction, fewer compounds could be identified under the conditions employed for separating the components of the PNA fraction.

Discussion

The Ames assay has been used extensively for mutagenic screening of pure chemicals (*11*); however its use for bioassay of complex chemical mixtures has been more limited. Cigarette smoke condensate (*12, 13*), complex mixtures of polycyclic compounds associated with airborne pollutants (*14, 15*), and to a lesser extent, synthetic fuels (*16*), have each been assayed in this way. In the work described here, a crude shale oil was tested for mutagenicity against two of the Ames test strains, *S. typhimurium* TA98 and TA100. In general, data for the mutagenic properties of this crude product and its major subfractions were in agreement with published data on synthetic crude oils (*16*), i.e., in the requirement for metabolic activation and in the location of major mutagenic activity in the basic and

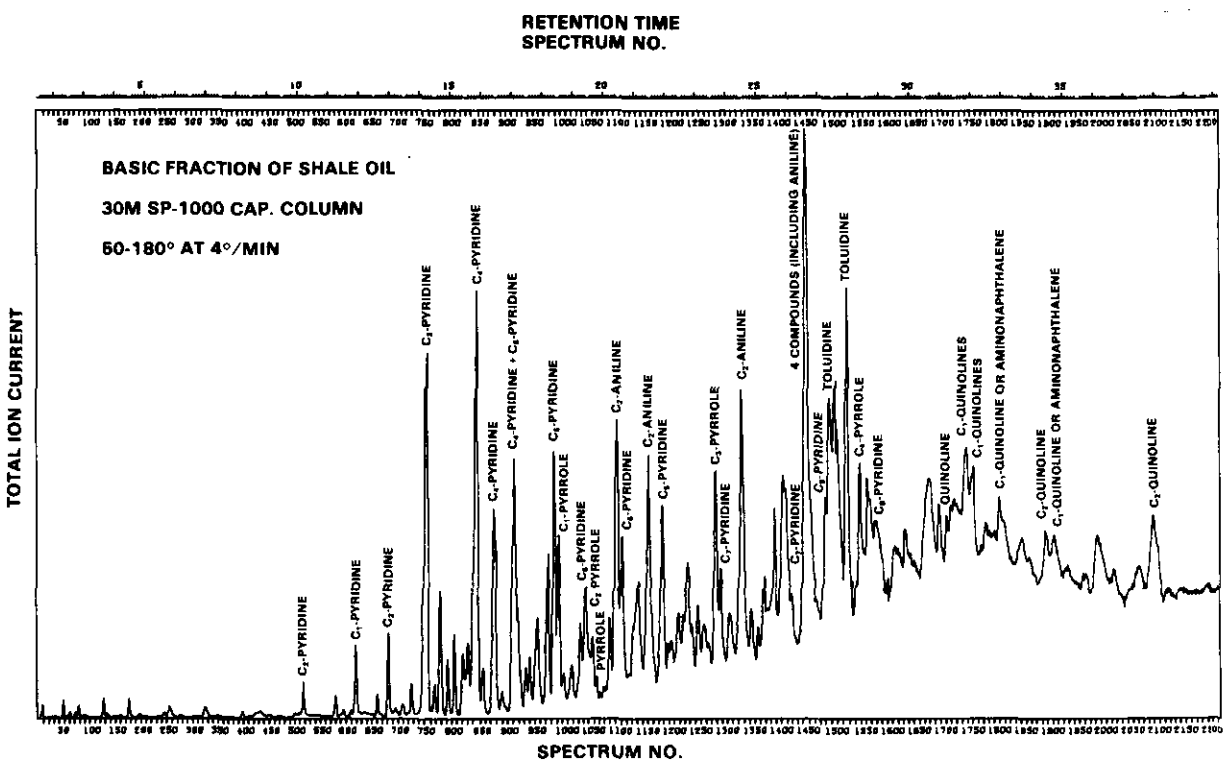

FIGURE 19. GCMS separation of components present in basic fraction of shale oil S-11. Column: 30M SP-1000 cap. column: temperature rise 50-180°C at 4°C/min.

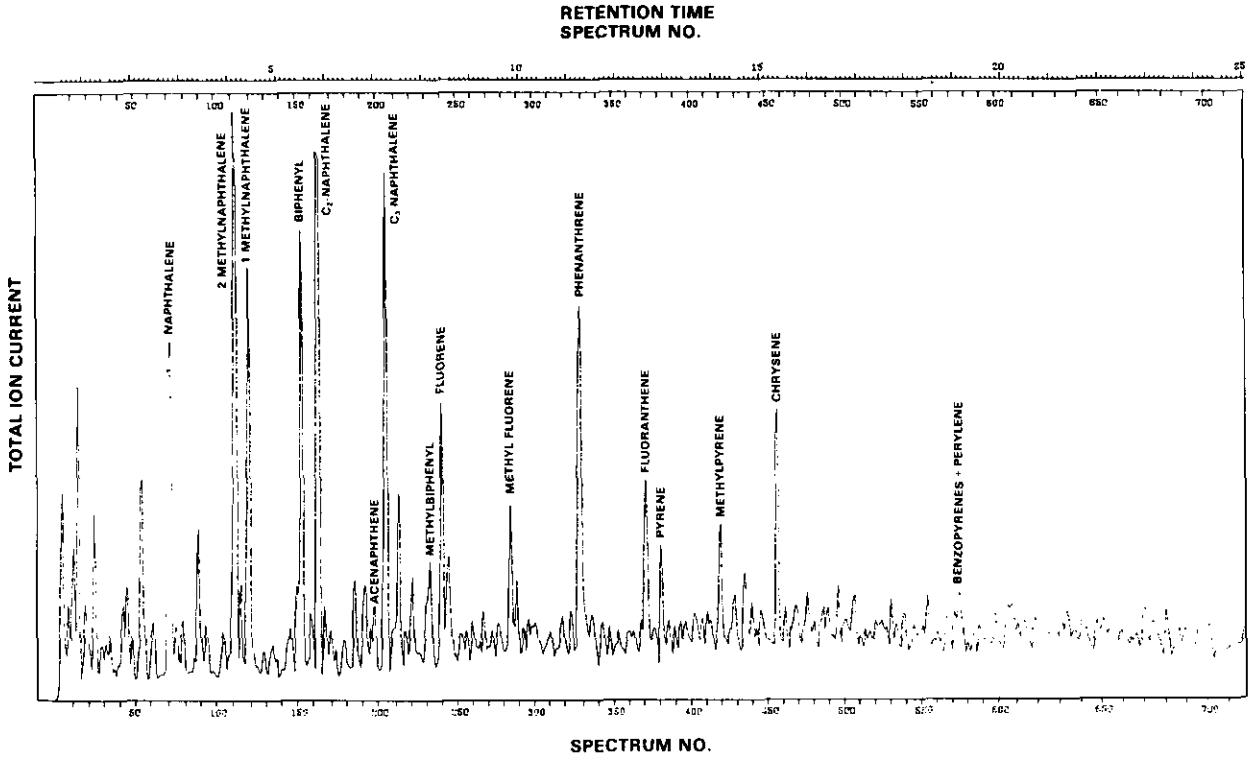

FIGURE 20. GCMS separation of components present in PNA fraction of shale oil (peak profile analysis). Column 6 ft 3:OV-17; temperature program 80-280°C at 8°C/min.

PNA fractions.

In quantitative terms, the most active of the shale oil fractions were consistently less mutagenic than reported values for comparable fractions from cigarette smoke condensate, i.e., approximately 1 revertant colony formed/μg (basic fraction, shale oil) vs. a maximum of about 18 revertants/μg condensate (basic fraction). On the other hand, the mutagenicity of the shale oil PNA fraction was closer to reported values for complex mixtures enriched in polycyclic hydrocarbons from polluted air, i.e., in the range 1–4 revertants/μg (*14, 15*).

As expected, identifiable components in the basic and PNA fractions from shale oil were chemically quite different. The basic fraction was rich in alkyl-substituted anilines, pyridines, pyrroles, and quinolines; many of the latter compounds have been identified as premutagens in the Ames assay (*17*). It is possible that premutagenic aminonaphthalenes were also present, although these compounds could not be separated from methyl quinolines, which may also have been present. The PNA fraction was rich in naphthalenes, biphenyls, and phenanthrenes, although more complex structures which corresponded to benzanthracene and benzopyrene were also observed.

The mutagenic properties of the basic and PNA fractions also differed. The PNA fraction appeared to mask much of the mutagenicity of benzopyrene, benzanthracene and dimethylbenzacridine. The level of apparent inhibition was greatest for the two polynuclear aromatic compounds (80–90%), and somewhat less for the acridine (about 75%). On the other hand, the PNA fraction did not significantly reduce the mutagenicity of 2-aminoanthracene. Accordingly, the bacterial test strain used in these experiments (TA100) did not lose its capacity to respond to a mutagen as a result of exposure to PNA fraction. Further, the enzymes for converting the primary amine into a mutagen remained functional in the presence of the PNA fraction. Thus, neither nonspecific killing of the test strain nor denaturation of the S9 enzymes are likely explanations for the inhibitory effects of the PNA fraction on the mutagenicity of benzopyrene and benzanthracene.

Similar mixing experiments using the basic fraction and the purified chemicals, 2-aminoanthracene and dimethlybenzacridine gave different results from the previous experiment. First, the mutagenicity of dimethylbenzacridine was only slightly reduced, judging by the response curves for this compound plus and minus basic fraction (i.e., 22%). The apparent mutagenicity of 2-aminoanthracene in a comparable experimental situation was more var-

ied. In most experiments, mixtures of this chemical plus basic fraction yielded a stronger mutational response than expected. For example, in the kinetics experiments reported, the number of revertant colonies obtained for the mixture (chemical + fraction) was as much as threefold greater than the sum of revertants for these materials assayed separately. However, in one set of experiments, the combination of 2-aminoanthracene plus basic fraction did not yield a higher number of revertant colonies than expected. The reason for the disparity of the two results is not known, but it may be related to slight variations in the selective medium and more importantly, to the fact that different S9 preparations were used in the two sets of experiments. (Catalytic activity of these different microsomal preparations was similar in terms of the number of revertant colonies obtained for 2-aminoanthracene; thus, large variations in the activity of the S9 preparations is not a likely explanation for the variability of the two sets of data.)

The number of revertants formed by addition of the test cells at various time intervals after initiation of the S9 reactions was used to estimate the effect of the complex fractions on the stability of these enzyme systems as employed in the standard Ames assay. For benzopyrene plus or minus the PNA fraction, the decay in mutagenic potential was almost complete within the first 100 min. Strong inhibition of mutagenesis was also observed for samples containing the mixtures of benzopyrene + fraction. This inhibition did not allow conclusions to be drawn about the relative stability of the S9 enzymes in the presence of the PNA fraction.

On the other hand, in similar experiments, the losses of mutagenic potential occurred at slower rates for 2-aminoanthracene or the mixture of 2-aminoanthracene plus basic fraction. Thus comparisons of S9 stability in the presence and absence of basic fractions could be made. In each of these cases, the decay of mutagenic potential was determined by the concentration of the aromatic amine, but not by the concentration of the basic fraction. At the lower levels of 2-aminoanthracene tested (i.e., corresponding to the midpoint of the response curves for this compound) the loss of mutagenic potential was fairly rapid (0–200 min). At higher concentration levels of 2-aminoanthracene, the lifetime of mutagen formation was extended and the decay rate reduced. It is clear from these data that addition of the basic fraction to the top agar did not lower the stability of the activating enzymes for 2-aminoanthracene, although the magnitude of the mutagenic response varied, as discussed above.

The preferential activation of the tar fraction in a liquid medium, as opposed to the standard Ames assay, is of interest. For the most part, pure chemicals showing microsomal-mediated mutagenicity are more easily activated in an agar medium, e.g., benzopyrene (*7*) and vinyl chloride (*18*). Relatively few chemicals, notably *N*-nitrosodimethylamine, are preferentially activated in a liquid medium (*19, 20*). As a practical device, it may be an advantage to carry out the activation reactions of complex mixtures in a liquid medium, especially in those instances where a high degree of cellular toxicity might result from long-term exposure of test cells at the concentrations required to cause mutagenesis.

Moreover, one must consider the possibility that half lives of metabolically derived mutagens in the agar gel of the standard Ames assay may not be the same as those generated by activation of mutagens in solution.

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