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Microbial Degradation of Petroleum Hydrocarbons: Implications for Arson Residue Analysis

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ABSTRACT: The effects of selective microbial activity on hydrocarbon accelerants have been examined both in vitro and in simulated arson residues. Two groups of *Pseudomonas* species that assimilate components of common petroleum based accelerants have been found; one species metabolizes only aromatic hydrocarbons, the other only aliphatics. The kinetics of hydrocarbon metabolism are discussed and gas chromatographic profiles of two common accelerants that have been subjected to microbial attack are presented. Some implications and recommendations for arson investigation are discussed.

KEYWORDS: forensic science, microbial degradation, gasoline, accelerants, arson residues, pseudomonads, pseudomonas putida, pseudomonas biovarIII

Our laboratory processes many hundreds of arson residues per year using the headspace sampling method described previously [*1*]. Residues are normally presented to our laboratory in sealed paint tins and might spend a period of between one and three weeks in storage before analysis. In a small but significant number of cases unusual mixtures of hydrocarbons are detected. Figure 1 is a gas-liquid chromatogram of such a mixture; as can be seen there are many similarities between this chromatogram and that of authentic gasoline (Fig. 2) but the absence of key substances such as benzene, toluene or hexane made conclusive identification for forensic purposes difficult.

It was assumed that these anomalous hydrocarbon mixtures originated from common accelerants and it was clear that they were not the products of simple evaporative or combustive concentration. If the accelerants had been concentrated by either of these mechanisms then the mixtures produced would have been richer in compounds of low volatility. Such a pattern was not observed. It can be seen from Fig. 1 that toluene, for example, appears to have been removed from the accelerant (gasoline) but 3-methylhexane, a much more volatile substance, has not. It appeared that a complex mechanism was in operation whereby certain compounds were removed selectively from the accelerant.

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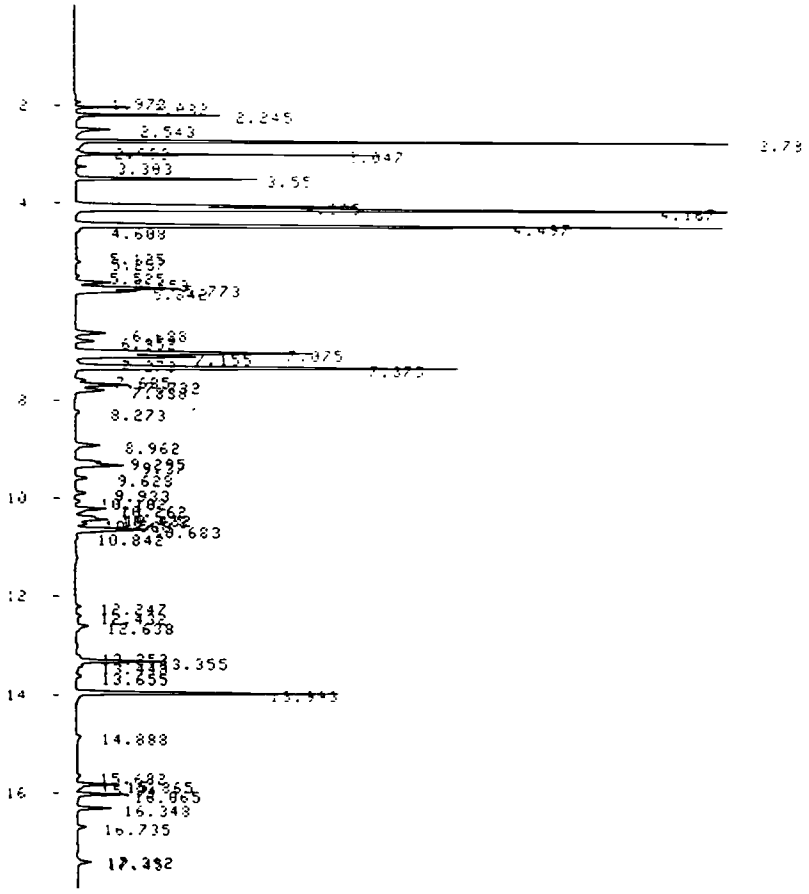

FIG. 1—*Gas chromatogram of an anomalous mixture of hydrocarbons detected in an arson residue.*

In a few instances it was found that the arson residue itself was responsible for causing anomalous chromatograms; the matrix selectively entrained aromatic hydrocarbons. Steam distillation or solvent extraction of these residues freed the aromatics and provided readily identifiable accelerant chromatograms. In many instances, however, anomalous mixtures were detected and steam distillation or solvent extraction of the residue failed to produce an identifiable accelerant. It was thought that these mixtures were common accelerants that had been modified by microbial attack; this supposition was reinforced by the work of Mann [*2*].

The aim of the investigation reported here was to examine the effects of microorganisms on common hydrocarbon accelerants, such as gasoline, both in vitro and in matrices similar to those encountered as arson residues.

Methods

The sources of microorganisms for this study were two genuine arson residues that were found to give anomalous gas chromatograms. Samples of soil were taken from these residues and inoculated into enrichment cultures containing Stanier's mineral salts medium (minimal medium, 100 mL, see Experimental) and an aliquot (0.1 mL) of the

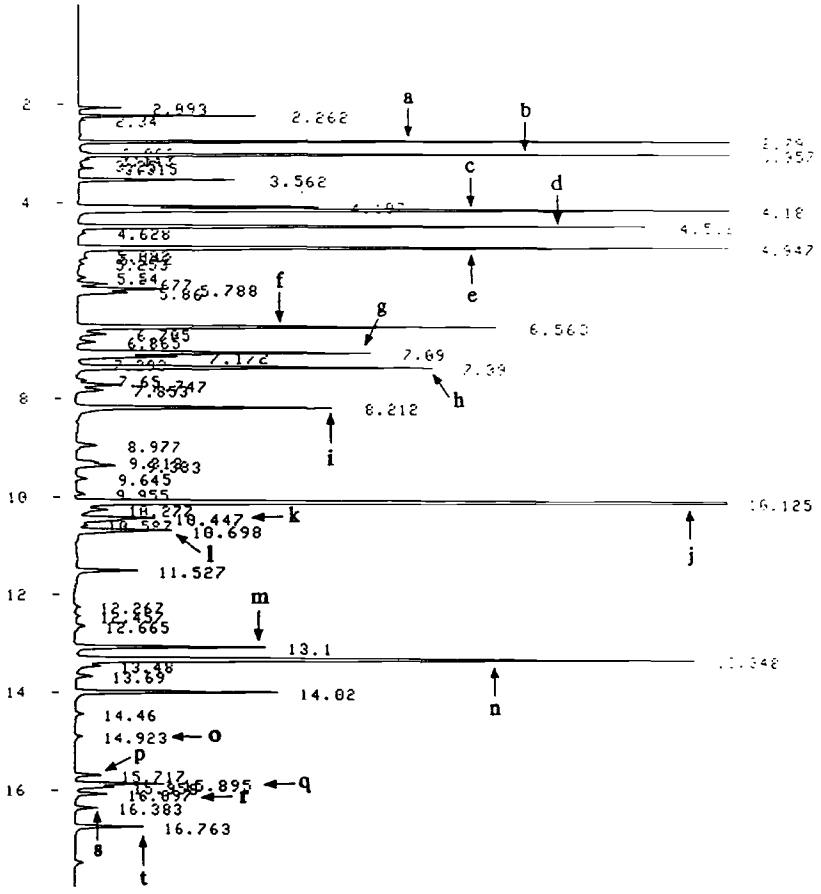

FIG. 2—*Gas chromatogram of headspace above gasoline. The letters marked on the chromatogram refer to the following substances: a = 2-methylbutane, b = pentane, c = 2-methylpentane, d = 3-methylpentane, e = hexane, f = benzene, g = 2-methylhexane, h = 3-methylhexane, i = heptane, j = toluene, k = 2-methylheptane, l = 3-methylheptane, m = ethylbenzene, n =* m- *and* p-*xylene, o =* iso-*propylbenzene, p =* n-*propylbenzene, q =* m- *and* p-*ethyltoluene, r = 1,3,5-trimethylbenzene, s = o-ethyltoluene and t = 1,2,4-trimethylbenzene.*

putative hydrocarbon accelerant. In one residue the accelerant appeared to be gasoline, in that case unleaded gasoline was used in the enrichment medium. In the other residue petroleum naphtha[4] was the putative accelerant so it was used in the enrichment medium.

The inoculated samples were then incubated at 25°C for 7 days on a shaking table. After this time a loopful of each enrichment culture was subcultured into fresh enrichment media and incubated as before. These subcultures were then plated out onto Stanier's minimal agar (Experimental) and nutrient agar (Experimental) and incubated for up to 7 days. From these plates seven pure cultures were obtained; four from enrichment in gasoline (referred to below as microorganisms Pp#8, Pp#11, Pp#12 and Pp#13) and three from shellite (referred to below as microorganisms Pf#1, Pf#2 and Pp#4).

[4]Petroleum naphtha is a very volatile hydrocarbon fuel low in aromatic hydrocarbons and rich in aliphatics, its main use is in camping stoves and appears to be called "Coleman Fuel" or "White Gas" in the USA. It is referred to by the Australian trade name "shellite" hereafter.

The seven microorganisms isolated were all found to be Gram-negative bacteria. Identification was carried out using the Microbact 24E system standard kit and additional biochemical tests including the production of pyocyanin, fluorescein, lecithinase, levans, enzymes for the hydrolysis of gelatin and starch, denitrification, and growth at 41°C. Microorganisms Pp#8, Pp#11, Pp#12, Pp#13 and Pp#4 were identified as different strains of *Pseudomonas putida* while Pf#1 and Pf#2 were identified as *Pseudomonas fluorescens biovarIII*. The conditions under which the cultures were enriched would encourage the growth of pseudomonads at the expense of other microorganisms. It is therefore not surprising that only pseudomonads were isolated from the two arson residues.

Inocula of each of the purified organisms were prepared by culturing them in nutrient medium (20 mL, see Experimental) mixed with appropriate hydrocarbon fuel (either gasoline or shellite, 0.1 mL). These mixtures were incubated at 25°C for 7 days then 0.1 mL of each of the cultures resubcultured into fresh nutrient medium/fuel mixture. For experiments using minimal medium, the inocula were prepared using Stanier's minimal medium instead of nutrient medium.

The experiments conducted in vitro were performed by adding 0.1 mL of inoculum to 20 mL of liquid growth medium (either Stanier's or nutrient media) and 0.1 mL of hydrocarbon fuel (gasoline or shellite) in a 300 mL biological oxygen demand (BOD) bottle as shown in Fig. 3. The bottles, after inoculation, were sealed with silicone grease and water, covered with a plastic cap, and incubated at 25°C on a shaking table. The water seals were replaced at regular intervals to prevent evaporative loss of the hydrocarbon fuel and to ensure that foreign microorganisms were excluded. Control experiments were established by adding 0.1 mL of 0.1% $HgCl_2$ solution to the growth medium/fuel mixture; inoculum was not added. At appropriate time intervals incubated samples were subjected to gas-liquid chromatographic (GLC) analysis in an effort to establish whether any components of the fuel had been assimilated by the organism. To accomplish this the seal on the BOD bottle was broken and 0.25 mL of the headspace was withdrawn.

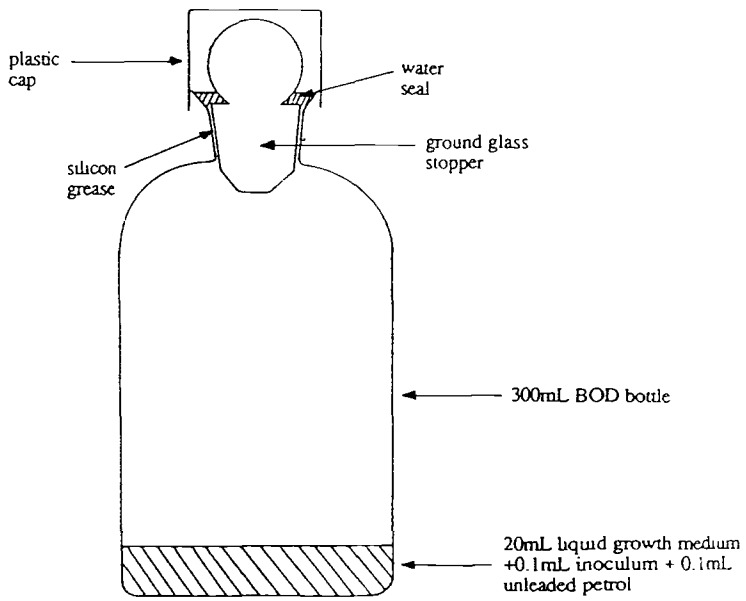

FIG. 3—*Biological oxygen demand (BOD) bottles as used in Experiments.*

Headspace samples were then subjected to cryogenically focussed GLC using a modification of the method described by Kobus and Kirkbridge [*1*] (see Experimental); the gas chromatograph was a Varian model 3400 equipped with a flame ionization detector, N_2 was carrier gas at a mean flow of 20 cm/sec, a 25 m × 220 μm × 1.0 μm BP1 fused silica capillary column was used with a temperature program of 45°C (5 min) – 175°C at 8°C/min.

The extents to which hydrocarbons in fuels were assimilated by microorganisms were calculated from data produced by a Shimadzu Chromatopac C-R6A electronic integrator. Experience with case samples, and subsequent experiments, indicate that some hydrocarbons present in fuels are not assimilated at all and some only extremely slowly. In petrol, for example, 2-methylhexane, 3-methylhexane, 2-methylheptane, 3-methylheptane, *m*-, *p*- and *o*-xylene, 1,3,5-trimethylbenzene and 1,2,4-trimethylbenzene are not readily assimilated by *P. putida*. Chromatographic peak areas due to these hydrocarbons were used as "internal standards" against which were measured the levels of benzene, toluene, ethylbenzene and *n*-propylbenzene remaining after exposure of petrol to *P. putida*. As well as being resistant to attack by microorganisms, the standards are very close in volatility to these aromatics. Therefore if any evaporative loss of these aromatics was to occur during incubation, it should be accompanied by an equivalent evaporative loss of the internal standards. Quantitative data relating to the consumption of simple aliphatics in shellite were obtained in an analogous fashion with 2-methylbutane, 3-methylpentane, cyclohexane, 3-methylhexane, 2,4-dimethylhexane, and 3-methylheptane as internal standards. Identification of hydrocarbon components in fuels was achieved using either gas chromatography-mass spectrometry, or by subjecting fuels spiked with hydrocarbon standards to GLC. Figures 2 and 8 show the retention data for the internal standards.

After GLC the cultures were checked for purity on nutrient agar.

The experiments designed to imitate microbial degradation in arson residues were performed in the following manner. Inoculum (0.5 mL), prepared in the presence of gasoline, was added to mixtures of sterile soil (250 g), Stanier's minimal medium (50 mL) and unleaded gasoline (1 mL) in clean 4L paint tins. Controls were prepared by omitting the inoculum. After a predetermined time, 1 mL of headspace was withdrawn from the tin and subjected to GLC as above.

Results and Discussion

The activity of each of the seven microorganisms was examined by culturing each with samples of unleaded gasoline in either minimal medium or nutrient medium; a control, prepared without inoculation, was also set up. Microbial growth was evident in both media within 48 h but not in the control. Gas liquid chromatography of the headspace above each sample showed that, compared to the control sample, levels of certain hydrocarbons had been reduced in all samples. This experiment showed that the individual strains of each species of microorganism behaved in a similar fashion. Further experiments were therefore conducted using only one representative of each species (Pp#11 and Pf#2).

Figure 4 shows the chromatogram produced by headspace analysis of unleaded gasoline that had been exposed to microorganism Pp#11 in minimal medium. The arrows along the baseline indicate the position of peaks present in the control that are not present in the inoculated sample. Position A is where benzene elutes, B toluene, C ethylbenzene, D *iso*-propylbenzene and position E *n*-propylbenzene. A similar chromatogram was produced by the headspace above unleaded gasoline and organism Pp#11 in nutrient medium except that peaks due to benzene and monosubstituted aromatics were not completely absent. Assimilation of aromatic hydrocarbons was more rapid in minimal medium be-

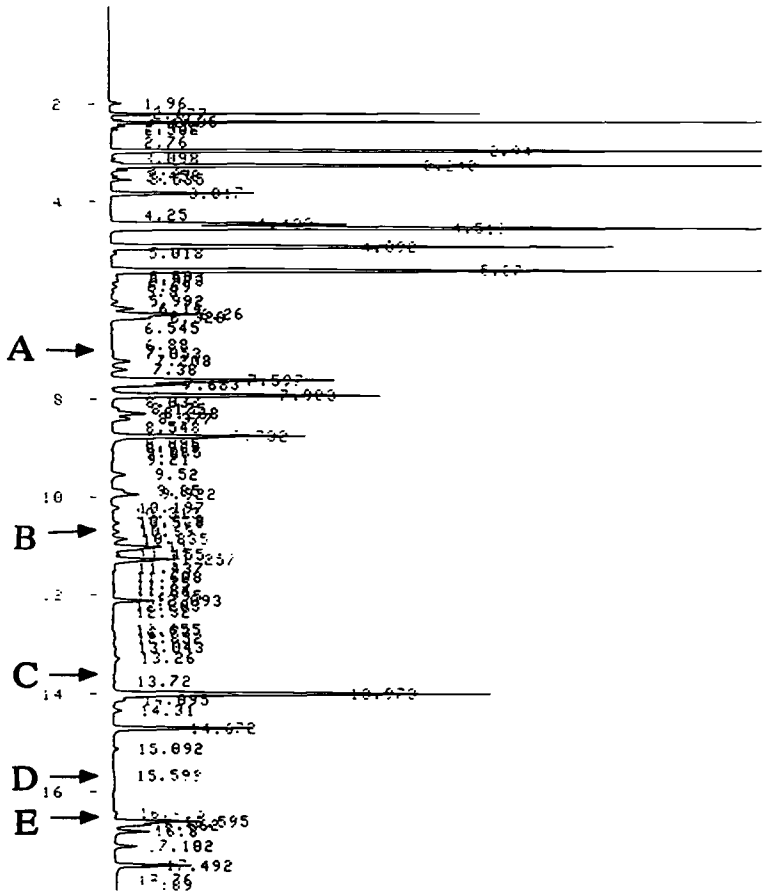

FIG. 4—*Headspace chromatogram of gasoline exposed to microorganism Pp#11 in minimal medium. The positions marked on the chromatogram are where the following substances elute: A = benzene, B = toluene, C = ethylbenzene, D =* iso*-propylbenzene and E =* n*-propylbenzene.*

cause the fuel was the sole source of carbon and energy for the microorganisms. On the other hand, in nutrient medium the microorganisms have a choice of various carbon sources, hence the less rapid metabolism of the fuel. All subsequent trials in vitro were therefore conducted in minimal medium.

The residue from the inoculated minimal medium was extracted using dichloromethane. Analysis of this extract using GLC showed that benzene and the monosubstituted aromatic substances were not present (see Fig. 5). It was therefore confirmed that these substances have indeed been metabolized and not entrained by the growth medium, the microorganisms or the apparatus.[5]

It was apparent that microorganism Pp#11 did not metabolize the aliphatic components of gasoline in the presence of aromatics. Indeed, experiments using pure aliphatic hydrocarbon standards (pentane, hexane, heptane and octane) showed that none of the strains of *P. putida* could use these substances.

[5]In initial experiments rubber seals were used instead of silicone grease and water. Rubber was found to remove aromatics selectively—but reversibly—from the inoculated medium, giving rise to chromatograms similar to Fig. 1.

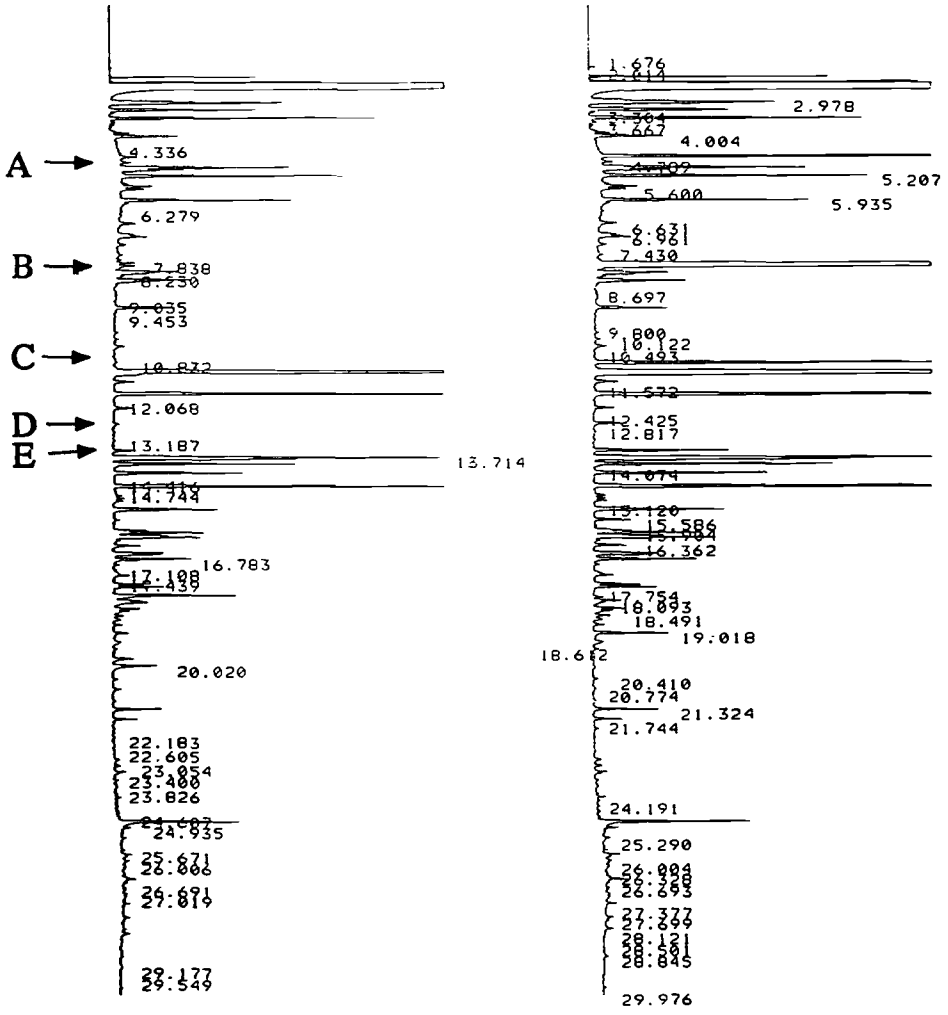

FIG. 5—*Right: Dichloromethane extract of minimal medium and gasoline mixture (control). Left: Dichloromethane extract of minimal medium and gasoline mixture exposed to microorganism Pp#11. The positions marked on the chromatogram are where the following substances elute: A = benzene, B = toluene, C = ethylbenzene, D =* iso*-propylbenzene and E =* n*-propylbenzene.*

Microorganism Pp#11 was inoculated into ten samples of minimal medium mixed with unleaded gasoline. At appropriate time intervals, over a period of 48 h, a sample was selected and subjected to headspace GLC analysis; after 48 h the control was examined. From the GLC data were calculated the levels of benzene, toluene, ethylbenzene and *n*-propylbenzene remaining at each time interval as a percentage of the amounts present in the control. When plotted against time the concentration values yield curves depicting rates of consumption of aromatic hydrocarbons; see Fig. 6. The plots show that benzene, toluene and ethylbenzene are assimilated in an exponential fashion after an induction period of about 15 h. Complete consumption of these aromatics is evident after 30 to 40 h. The level of toluene does not appear to reach 0%; it is thought that the peak being measured after 30 h is actually a substance that is not degraded and coelutes with toluene. From the plots it appears that substituents larger than a methyl group retard assimilation

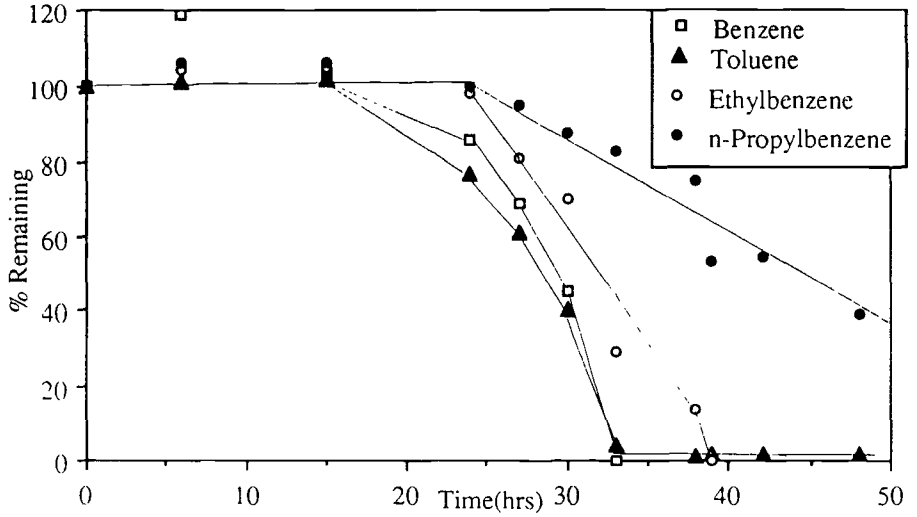

FIG. 6—*Rates of consumption of aromatic hydrocarbons in gasoline exposed to microorganism Pp#11 in minimal medium.*

of an aromatic hydrocarbon. This phenomenon has been reported previously by Scow [*3*] and Gibson [*4*].

The effects of prolonged exposure of gasoline to microorganism Pp#11 were investigated over a period of 6 weeks. In addition to benzene, toluene, ethylbenzene and *n*-propylbenzene, polysubstituted aromatics such as the xylenes, the ethyltoluenes and trimethylbenzenes were also degraded—albeit slowly. Figure 7 shows the chromatographic region over which these C8 and C9 aromatic hydrocarbons in gasoline elute and the effects on peak intensities that prolonged exposure has. Table 1 summarizes the relative rates of degradation of hydrocarbons in gasoline exposed to microorganism Pp#11.

Six samples of minimal medium were treated with shellite and inoculated with microorganism Pf#2; a control was also set up. Figures 8 and 9 are headspace chromatograms from the control (unchanged shellite) and shellite degraded by the microorganism over a period of 6 days, respectively. The levels of alkanes were measured at 24 hourly intervals and a plot of this data is presented as Fig. 10.

Three important points have emerged. Firstly, consumption of toluene and benzene by microorganism Pf#2 is negligible. However, its ability to degrade aliphatics is substantial and it is apparent that this isolate has enzymes for the metabolism of aliphatics but not aromatics. The isolates of *P. putida*, on the other hand, evidently only possess enzymes capable of degrading aromatics and not aliphatics. The two species of microorganisms therefore exhibit complimentary behavior.

Secondly, the absolute rate of assimilation of aliphatic substances by organism Pf#2 is slower than rate of consumption of aromatics by *P. putida* isolates.

Thirdly, there is some evidence of microbial selectivity between the aliphatic hydrocarbons. Unbranched alkanes are consumed relatively rapidly. The 2-methylalkanes are consumed less rapidly and the rate of consumption of these branched alkanes increases with increasing chain length. These findings are in accord with those of Bartha and Atlas [*5*], Dias and Alexander [*6*], and Hammond and Alexander [*7*].

Some case samples submitted for analysis showed evidence of complex microbial attack, that is, consumption of both aromatic and aliphatic hydrocarbons; the cases discussed by Mann [*2*] also appear to feature this kind of degradation. It is possible that in those

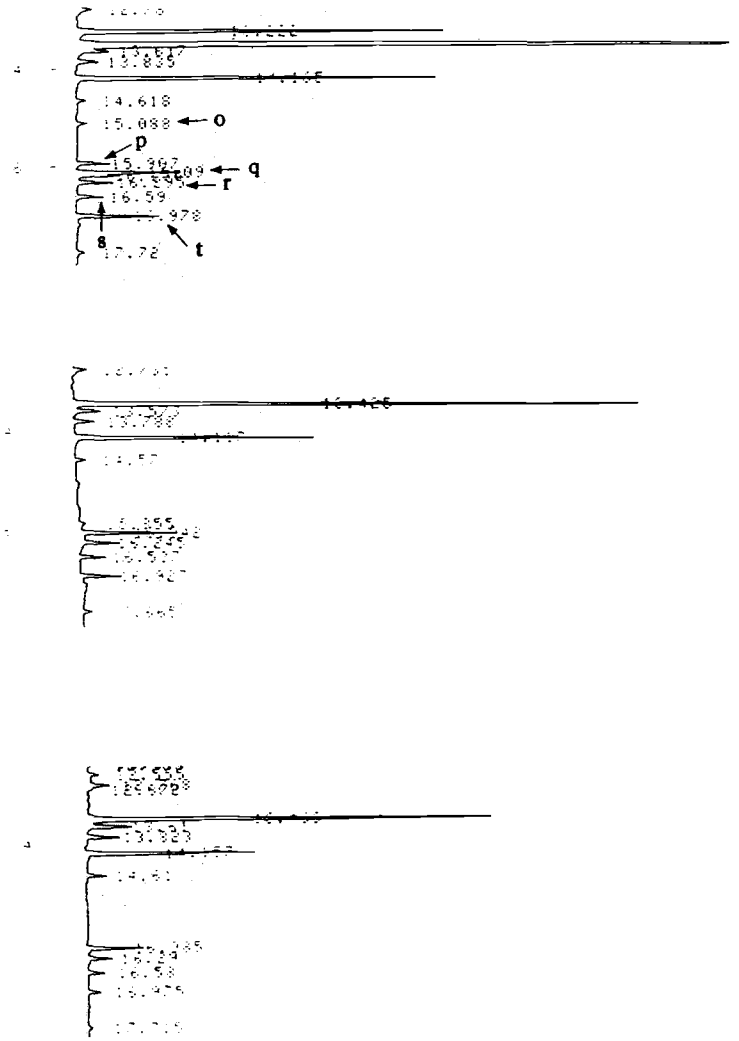

FIG. 7—*Effects of prolonged exposure of petrol to microorganism Pp#11. Top: Headspace above a mixture of gasoline and minimal medium after 6 weeks (control). Middle: Headspace above gasoline exposed to microorganism Pp#11 in minimal medium for four weeks. Bottom: Headspace above gasoline exposed to microorganism Pp#11 in minimal medium for six weeks. The peak assignments are as given in Fig. 2.*

cases a mixed culture containing *Pseudomonas putida* and *P. fluorescens biovarIII* might have been present. Alternatively, a microorganism different to those isolated in this study might have been responsible. As the possibility that an arson residue might contain a mixed culture is high, an assimilation experiment using a mixed culture was conducted. Aliquots of minimal medium mixed with either shellite or unleaded gasoline were treated with a mixed inoculum containing organisms Pp#11 and Pf#2. Figure 11 is the chromatogram of headspace above unleaded gasoline that had been exposed to the mixed inoculum. Aliphatic and aromatic substances were assimilated in each fuel. The combined effect of the two organisms appeared to be a combination of their behaviors in isolation.

TABLE 1—*Relative rates of degradation of hydrocarbons.*

Rate	Hydrocarbon
Primary degradation (within 7 days at 25°C)	benzene toluene ethylbenzene n-propylbenzene iso-propylbenzene
Secondary degradation (within 1–6 weeks incubation at 25°C)	m- & p-xylene o-xylene 1-methyl-4-ethylbenzene 1,2,4-trimethylbenzene 1-methyl-3-ethylbenzene 1,3,5-trimethylbenzene 1-methyl-2-ethylbenzene
Inert to degradation (>6 weeks incubation required)	aliphatics & alicyclics

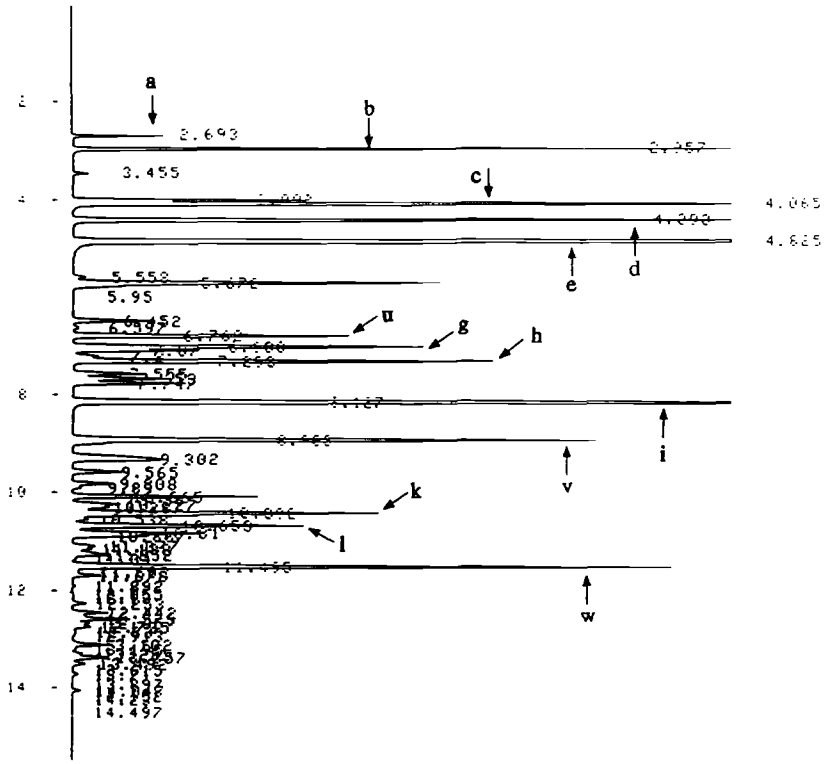

FIG. 8—*Headspace above a mixture of shellite and minimal medium (control). The peak assignments are as given in Fig. 2 with peak "u" being due to cyclohexane, "v" = 2,4-dimethylhexane and peak "w" = octane.*

As observed with the pure cultures, the aromatic hydrocarbons were metabolized much more rapidly than the aliphatics.

An ideal environment for the growth of microbes was provided in the in vitro experiments but these conditions might not be available to organisms present in an arson residue. In an effort to examine microbial degradation under conditions resembling those

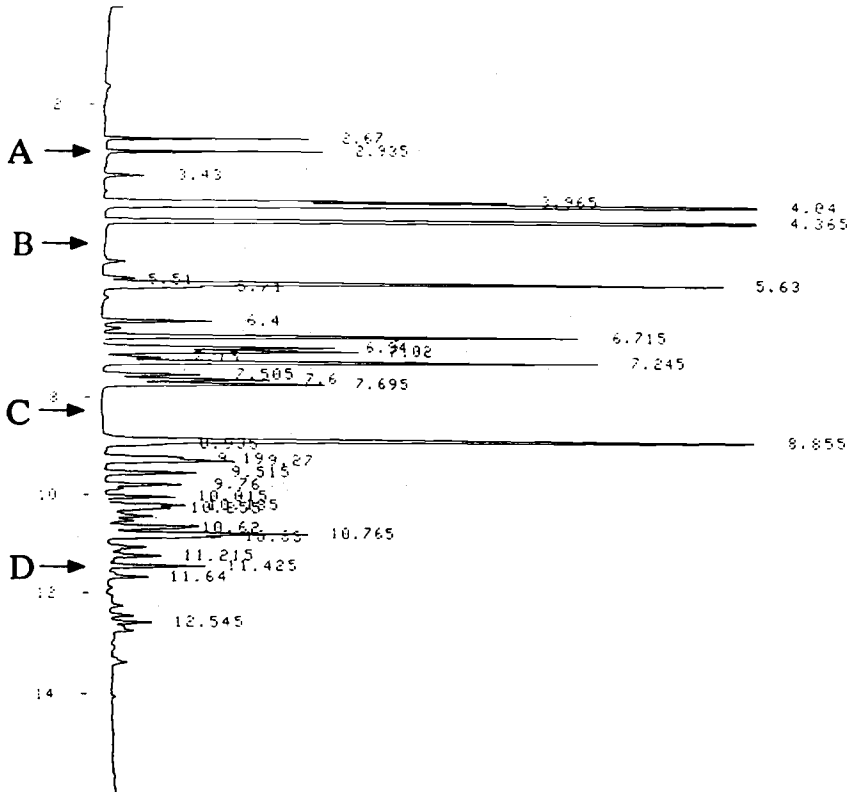

FIG. 9—*Headspace above shellite exposed to microorganism Pf#2 in minimal medium for six days. The positions marked on the chromatogram are where the following substances elute: A = pentane, B = hexane, C = heptane and D = octane.*

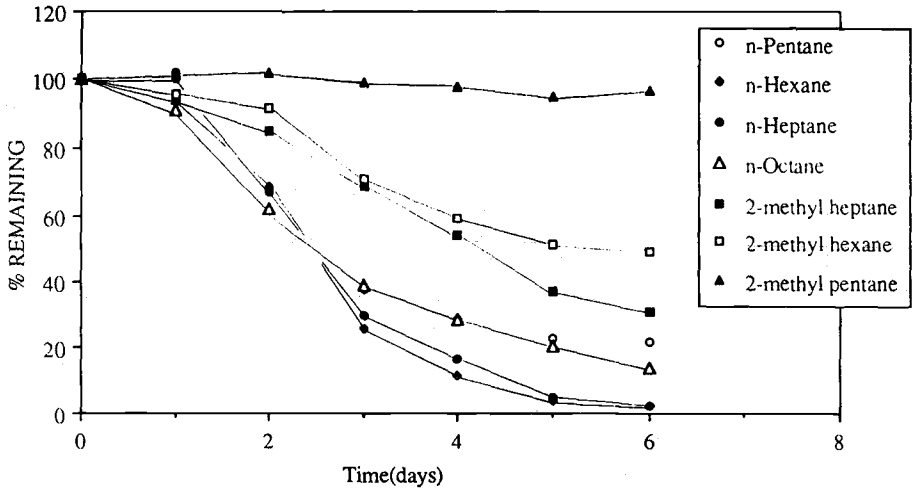

FIG. 10—*Rates of consumption of aliphatic hydrocarbons in shellite exposed to microorganism Pf#2 in minimal medium.*

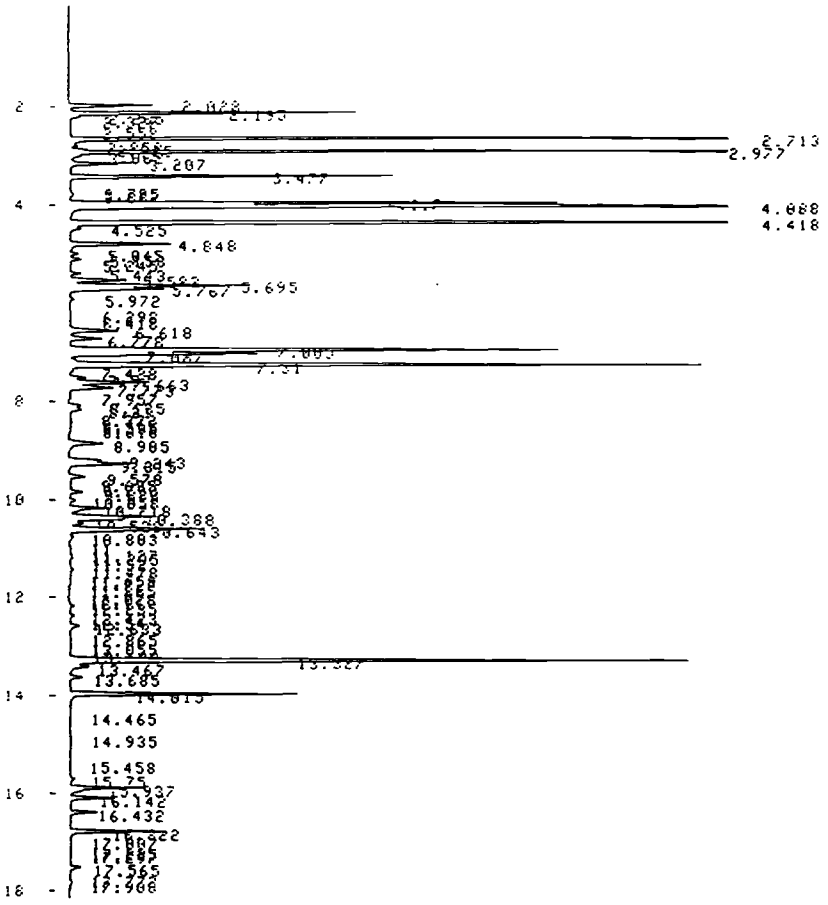

FIG. 11—*Headspace above gasoline exposed to a mixed inoculum of microorganisms Pp#11 and Pf#2 in minimal medium.*

in casework samples, mixtures of minimal medium (50 mL), sterile soil (250 g) and unleaded gasoline (1 mL) were inoculated with microorganism Pp#11. A control was prepared by adding petrol to sterile soil and minimal medium. In a manner identical to that observed in the in vitro experiments, organism Pp#11 consumed only benzene and monosubstituted aromatics. It would appear that the in vitro experiments are a valid way of duplicating microbial activity in a diffuse, heterogeneous matrix like an arson residue.

One grade of Australian high-octane gasoline contains tetraethyl lead at a level that might be toxic to microorganisms. Over a period of seven days organisms Pp#8, Pp#11, Pp#12 and Pp#13 were all observed to metabolize the aromatic components of leaded gasoline. It therefore appears that the concentration of lead present in leaded spirit is not sufficient to inhibit microbial degradation of hydrocarbons.

Conclusion

It has been demonstrated that microbial action on accelerants is of importance to arson investigation. Certain species of *Pseudomonas* microorganisms have been shown to metabolize aromatic and simple aliphatic hydrocarbons that are important compounds in

the identification of common accelerants. Complete metabolism of benzene and mono-substituted aromatic substances was observed to occur within 48 h while simple aliphatic substances are degraded more slowly. Accelerants exposed to these microorganisms can be degraded almost beyond recognition, certainly beyond conclusive identification for forensic purposes.

The pseudomonads are ubiquitous organisms that are quite likely to be encountered in arson residues. Although a fierce fire is likely to sterilize a scene, unavoidable activities such as quelling the fire with water or exploring the scene with muddy boots can easily inoculate it.

To the analyst two courses of action are available. One is to ensure that microbial activity in an arson residue is kept to a minimum. Suitable steps might be to add a nonvolatile bactericide to arson residues or, as proposed by Mann [2], keep the items under refrigeration from the time they are collected until they are analyzed. The second approach is to recognize the effects microbial activity has on accelerant chromatographic profiles and then be prepared to demonstrate that an anomalous residue does indeed contain microorganisms that are capable of metabolizing hydrocarbons. It is our suggestion that in vitro tests as described above can be convenient for such a demonstration.

Experimental

Stanier's Minimal Medium

A solution of potassium nitrate (0.5 g), dipotassium hydrogen orthophosphate (0.1 g), magnesium sulfate (hydrate, 0.05 g), calcium chloride (hydrate, 0.05 g) and ferric chloride (hydrate, 0.01 g) were dissolved in distilled water (1 L).

Stanier's Minimal Agar

Salts as described for Stanier's minimal medium were dissolved in distilled water (500 mL). This solution was then mixed with a solution of agar (20 g) in distilled water (500 mL).

Nutrient Medium

This medium was prepared by adding nutrient broth #2 (25 g) to distilled water (1 L).

Nutrient Agar

Blood agar base (40 g) was dissolved in distilled water (1 L).

Cryogenically Focussed GLC

Cryogenic focussing was performed using an accessory as shown in Fig. 12 and not in a bath of acetone and dry ice as reported previously [1]. Connected to a split/splitless injector is a short piece (about 30 cm) of megabore capillary column (0.53 mm internal diameter, BP1 stationary phase) to which is attached the analytical GLC column by means of a glass push-fit union. Surrounding the megabore column is a short sleeve (120 mm) made of 1/16 inch stainless steel tubing equipped with inlet and outlet ports. This sleeve is held in place on the megabore column by conventional GLC ferrules and was custom manufactured by Scientific Glass Engineering Inc. of Melbourne, Australia. The sleeve and the megabore column can be cooled by a stream of nitrogen which comes from a heat exchanger immersed in liquid nitrogen. The flow of cold nitrogen is turned on for 25 s to reduce the temperature of the widebore column to approximately −60°C.

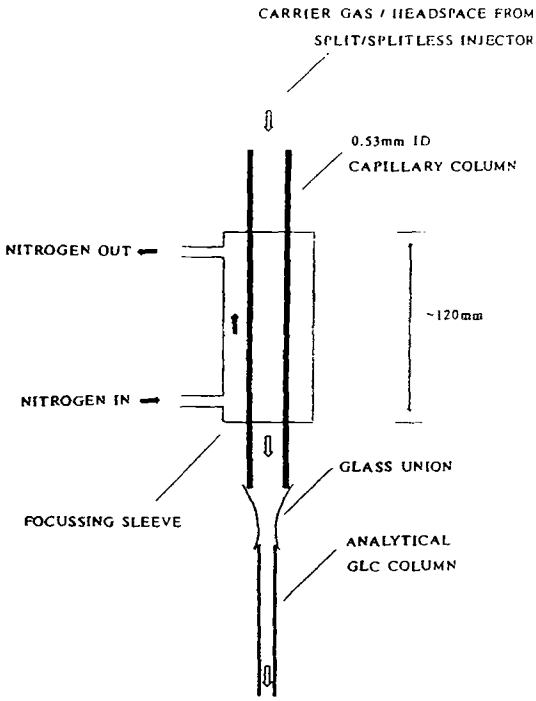

FIG. 12—*Schematic diagram of modified cryogenic focussing device as used in gas chromatographic analyses.*

With the cooling gas still flowing the headspace sample is then introduced and cryogenic focussing performed for 10 s. After this time the flow of cold nitrogen is stopped and the section of megabore column is allowed to reach column operating temperature ballistically. Substances focussed by the process are then free to chromatograph in the conventional manner. This technique greatly improves chromatographic peak shape, particularly when headspace samples of large volume or those produced by thermal desorption methods are analyzed.

Acknowledgment

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