Whole Microorganisms Studied by Pyrolysis-Gas Chromatography-Mass Spectrometry: Significance for Extraterrestrial Life Detection Experiments

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Pyrolysis-gas chromatography-mass spectrometric studies of two microorganisms, Micrococcus luteus and Bacillus subtilis var. niger, indicate that the majority of thermal fragments originate from the principal classes of bio-organic matter found in living systems such as protein and carbohydrate. Furthermore, there is a close qualitative similarity between the type of pyrolysis products found in microorganisms and the pyrolysates of other biological materials. Conversely, there is very little correlation between microbial pyrolysates and comparable pyrolysis studies of meteoritic and fossil organic matter. These observations will aid in the interpretation of a soil organic analysis experiment to be performed on the surface of Mars in 1975. The science payload of this landed mission will include a combined pyrolysis-gas chromatography-mass spectrometry instrument as well as several "direct biology experiments" which are designed to search for extraterrestrial life.

The analysis of microorganisms by pyrolysis-gas chromatography has recently become a rapid and reliable method for the characterization of potential pathogens (13–15). Even closely related strains of the same microorganism have been distinguished under controlled conditions (16). The method has also been extended to the diagnosis of viral and fungal diseases in plants (10). The identity of a particular organism is generally determined from an analysis of the pyrolysis "fingerprints," without identification of the individual pyrolysis products. However, in one study a small number of low-molecular-weight compounds were identified and reported to be present in the pyrolysates of several different microorganisms (12).

The NASA has recently announced that a combined pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS) experiment will be included on the first landed mission to Mars in 1975 (22). This experiment is intended to perform an organic analysis of the Martian soil with particular emphasis on the recognition of life-related organic compounds.

Martian life, if any, is considered to be synonymous with microorganisms. This view is so widely held that all of the four "direct biology" experiments, also to be included in the Mars mission, are experiments which seek to measure some specific property of microbial metabolism (4–6, 9, 23).

Since the Py-GC-MS experiment is designed to complement the biology experiments, it is important that a definite correlation exist between the pyrolysis products of microorganisms and those which may be liberated during the thermal degradation of soils.

For this investigation, two microorganisms, Micrococcus luteus and a common soil bacterium, Bacillus subtilis var. niger, have been pyrolyzed at 500 °C, their pyrolytic fragments separated by efficient capillary gas chromatography, and their individual compounds identified by mass spectrometry.

MATERIALS AND METHODS

Previous studies (18, 19) with the Py-GC-MS technique established that most of the major classes of terrestrial bio-organic matter are thermally degraded to a characteristic series of pyrolysis fragments. For example, proteins and amino acids form a series of nitriles, whereas carbohydrates degrade to a series of aliphatic aldehydes, ketones, and furan derivatives.
with CH$_3$, CHO, or CH$_2$OH substituents. Similarly, porphyrins degrade to a series of pyroles; fats and waxes pyrolyze to a continuous series of unbranched alkenes and alkanes. Thus, when new materials are pyrolyzed, it is possible to compare the products with the library and assign them to one of the biological classes of compounds.

To summarize the analytical method, 2 mg of dry whole organisms was placed in a stainless-steel tube (inner diameter, 0.127 cm; length, 3 cm). The sample was then pyrolyzed by heating to 500°C in 15 sec in a helium atmosphere. The pyrolysate was separated on a capillary column (152 m by 0.05 cm) coated with 10% Carbowax 20M. The column was temperature-programmed from 50 to 200°C at a heating rate of 4 degree/min, and mass spectra of the eluted components were recorded with an EAI Quadrupole 300 mass spectrometer. Compounds were identified by comparison of their mass spectral fragmentation patterns with library reference spectra.

**Organisms.** The organisms used in this study were *B. subtilis* var. niger (ATCC 9372) and *M. luteus* (formerly *M. lysodeikticus*) (ATCC 4698) Flem. The *M. luteus* was obtained as a lyophilized preparation from Worthington Biochemical Corp. and grown on an agar medium by the submerged culture technique reported by Beers (1). *B. subtilis* var. niger was grown aerobically at 30°C in Trypticase Soy Broth and harvested during the stationary phase after sporulation had occurred. The cells were carefully washed with distilled water and then lyophilized.

**Geochemical samples.** Data from the pyrolysis of the microorganisms were subsequently compared with other pyrolysis results from the thermal degradation of soils, meteorites, and ancient sediments under similar conditions. The following samples, which are used for comparison, have been previously analyzed and reported in detail elsewhere (19, 20): (i) meteorites [Murray (Wiik type II), Mokoia (Wiik type III), and Pueblito de Allende (5)]; (ii) ancient sediments [Fig Tree Shale, South Africa (age, 3 X 10$^9$ years)]; (iii) desert soil (Thermal soil, Mojave Desert, Thermal, Calif.); and (iv) humic acid (freshly extracted from Illinois agricultural soil).

**RESULTS**

Chromatograms of the pyrolysis products of *M. luteus* and *B. subtilis* are shown in Fig. 1. Despite the fact that the two organisms are ge-

![Fig. 1. Gas chromatogram of the pyrolysates of *M. luteus* and *B. subtilis* var. niger. Numbered compounds are identified in Tables 1 and 2.](http://aem.asm.org/Downloaded from http://aem.asm.org/)
| Protein | Carbohydrate | Nucleic acid | Lipid | Porphyrin |
|---------|--------------|--------------|-------|-----------|
| Ethanenitrile (9) | Acrolein (1) | Acrylonitrile (9) | Acrolein (1) | Pyrrole (25) |
| Acrylonitrile (9) | Acetone (1) | Ethanenitrile (9) | Ethene (1) | Methyl pyroles (26, 27) |
| Propanenitrile (10) | Butanone (4) | Propanenitrile (10) | Propene (1) | Dimethyl pyroles (29, 31) |
| Butanenitrile (12) | Pentanone (8) | Butanenitrile (12) | Butene (1) | C₄ alkyl pyrrole (34) |
| 2 Methyl propanenitrile (13) | Propanal (1) | Pyrrole (29) | | C₄ alkyl pyroles (36, 37) |
| Methyl butanenitrile (15) | Methyl propanal (2) | | | |
| Methyl pentanenitrile (20) | Methyl butanal (6) | | | |
| Benzonitrile (30) | | | | |
| Phenylacetonitrile (39) | Furan (1) | | | |
| Tolunitrile (40) | 2 Methyl furan (3) | | | |
| Phenol (42) | Dimethyl furan (7) | | | |
| o-Cresol (41) | Furfural (24) | | | |
| p-Cresol (43) | Methyl furfural (28) | | | |
| Ethyl phenol (44) | Furfuryl alcohol (33) | | | |
| Xylenols (45, 46) | Methyl butadiene (5) | | | |
| Indole (47) | Benzene (7) | | | |
| Methyl indole (48) | | | | |
| Pyrrole (25) | | | | |
| Methyl pyroles (26, 29) | | | | |
| Methanethiol (1) | | | | |
| Methane (1) | | | | |
| Ethene (1) | | | | |
| Propene (1) | | | | |
| Butene (1) | | | | |
| Methyl propene (1) | | | | |
| Methyl butene (1) | | | | |
| Benzene (7) | | | | |
| Toluene (11) | | | | |
| Styrene (21) | | | | |
| Ethyl benzene (14) | | | | |
| M-xylene, p-xylene (15b) | | | | |
| o-Xylene (17) | | | | |
| Propyl benzene (16) | | | | |
| C₄ alkyl benzene (19) | | | | |
| Pyridine (18) | | | | |
| Methyl pyridine (22) | | | | |
| Dimethyl pyridine (23) | | | | |
| Ethylene oxide (1) | | | | |

* Acetamide (35), propionamide (38), and acetophenone (32) could not be readily assigned to the classes listed above.

* Numbers in parentheses refer to peak number on chromatogram.

* Mass spectra were recorded every 2 sec during elution of the initial chromatographic peak (numbered 1) which permitted identification of individual compounds in the mixture.

Numerically unrelated, there is a qualitative similarity in the overall pyrolysis patterns. The extent of this agreement is particularly evident once the identification of individual fragments is known. The various pyrolysis products which have been identified by mass spectrometry and which are common to both microorganisms are listed in Table 1. The table is arranged similarly to a scheme developed for desert soils (19) in which individual thermal fragments are assigned to those classes of bio-organic compounds from which they most likely originated. Where compounds are ascribed to more than a single class, then there is equal probability that either or both classes contributed to their formation. For example, pyrrole and methyl pyrrole can be formed from the thermal degradation of protein and porphyrins. However, the amount of pyrolysis product contributed by each biopolymer will depend on its relative concentration in the parent organisms.

Table 2 lists a smaller number of minor pyrolysis products which were only found in one or other of the organisms, e.g., 2-thiapropane was not
observed in the *M. luteus* pyrolysate even though this compound has an easily identifiable mass spectrum.

The major pyrolysis fragments of the two organisms contain either oxygen or nitrogen atoms and occasionally both. Thus acetamide is the most abundant fragment in both microbial pyrolysates. Other major fragments include pyroles, pyridines, phenols, and furan derivatives. The only major hydrocarbon fragments are benzene, toluene, and low-molecular-weight alkenes containing five or less carbon atoms. The following inorganic gases were detected in both pyrolysates but are not included in the tables: CO₂, H₂O, COS, NH₃, and H₂S.

A comparison of the pyrolysis products which have been identified in the two microorganisms with previous pyrolysis studies of various materials is summarized in Table 3. The distribution of pyrolysis products is arranged according to the predominant character of the pyrolysate; i.e., whether the fragments are mostly hydrocarbons, or contain heteroatoms such as oxygen, nitrogen, and sulfur.

**DISCUSSION**

It is clear from the data in Table 1 that the major classes of bio-organic matter in living systems are well represented in both microorganisms studied. Protein and carbohydrate constitute the bulk of the organisms both in terms of the total number of pyrolysis products and the observation that the major fragments are readily assignable to these two classes. Lipid fragments are not particularly evident, consistent with the reported low fat content of most bacteria (8). The identification of acetamide as the most abundant thermal fragment in both microbial pyrolysates is rather surprising. Certainly conversion of carboxylic acids to their ammonium salts and subsequent pyrolysis to the amide is one possible source of this material. Glutamine and asparagine may also be sources. The fact that propionamide is also a significant pyrolysis fragment provides additional evidence since the thermal degradation of glutamine is a reasonable pathway to propionamide. Yet there is one additional source of acetamide, and possibly propionamide, which may be more important. Bacterial cell walls comprise 20 to 35% of the dry weight of the eubacterial cell and universally contain a fundamental building block, acetyl muramic acid (17). Thermal fragmentation of this polymeric cell wall material at the points shown in Fig. 2 can yield several acetamide molecules for each acetyl muramic acid unit. Furthermore, pyrolysis of the glucose units would contribute furan derivatives to the pyrolysate, which were indeed found to be significant products. Poly-D-glutamic acid is reported to form true capsules around bacterial cells in the genus *Bacillus* (21) and this may also be a source of propionamide and acetamide under pyrolysis conditions.

The small number of pyrolysis fragments listed in Table 2, which are potentially unique to a particular organism, may reflect subtle biochemical differences. It is more likely, however, that

**TABLE 2. Pyrolysis fragments identified in one organism only**

| Micrococcus luteus | Bacillus subtilis |
|-------------------|-----------------|
| Methyl styrene (21a) | 2 Thiapropene (4) |
| Methyl pentanone (15a) | Butadiene (1) |
| Pyrazine (19a) | Methyl pentene (5a) |
|                    | Naphthalene (43a) |
|                    | Butane (1) |
|                    | Furly methyl ketone (25) |

* Numbers in parentheses refer to peak number on chromatogram.

**TABLE 3. Heteroatomic composition of biological and abiotic pyrolysates**

| Determination          | Predominant pyrolytic fragments | Percentage of pyrolysis fragments which contain various heteroatoms |
|------------------------|---------------------------------|---------------------------------------------------------------------|
|                        |                                 | Nitrogen | Oxygen | Sulfur|
| Desert soil (Thermal, Calif.) | Heteroatomic | 22  | 30  | 4   |
| Humus extract (Ill. agricultural soil) | Heteroatomic | 25  | 29  | 8   |
| *Micrococcus luteus* Bacillus subtilis var. niger | Heteroatomic | 39  | 31  | 7   |
|                       | Heteroatomic | 33  | 30  | 7   |
| Murray meteorite Pueblito de Allende meteorite ("unextractable indigenous fraction") | Hydrocarbon | <1  | 10  | 20  |
|                        | Hydrocarbon | NO* | NO  | 7   |
| Mokoia meteorite Fig Tree Shale (S. Africa) | Hydrocarbon | NO  | 11  | 12  |
|                        | Hydrocarbon | <1  | 3   | 5   |

* None observed.

**Fig. 2. Possible thermal degradation pathway for bacteria cell wall material to yield acetamide and propionamide.**
they result from the different conditions under which the two organisms were cultured, or, alternatively, were present only in trace amounts and therefore not detected in the particular organism considered.

There are some interesting observations when the microbial pyrolysates are compared with the data from the various geochemical samples as shown in Table 3. All of the contemporary biological materials pyrolyze to large numbers of oxygen- and nitrogen-containing fragments. Both the microbial and soil samples contain relatively few sulfur compounds such as low-molecular-weight mercaptans and inorganic gases such as carbonyl sulfide and hydrogen sulfide. These sulfur compounds are most likely formed from sulfur-containing amino acids. Therefore, biological materials when pyrolyzed yield predominantly heteroatomic fragments which strongly reflect the presence of the major biochemical classes, such as proteins and carbohydrates. By contrast, the pyrolysates of ancient sediments which have undergone considerable diagenesis and meteoric samples of extraterrestrial origin (3) produce almost no nitrogen fragments and are predominantly hydrocarbon in character.

It is particularly interesting to note that the Murray and Mokoia meteorites which are believed to be terrestrial contaminated (11) contain significantly more oxygen and nitrogen fragments than a recent fall, the Pueblito de Allende meteorite. The extent of terrestrial contamination of this particular meteorite has been estimated as less than 1 μg/g of organic matter (2). A large number of sulfur fragments in the meteoritic samples is possibly indicative of synthetic reactions during pyrolysis, due to the relatively high concentration of elemental sulfur in these samples.

Some other interesting correlations are obtained when a comparison is made between the number of identical fragments found in various samples. For example, 91% of the Thermal Desert soil fragments were also observed in the pyrolysate of M. luteus. Similarly there was 87% agreement between Thermal Desert soil and humus acid extract. Correlations between meteorite samples were equally good; e.g., 92% of the Mokoia fragments were found in Murray. However, when biological and meteoritic samples are similarly compared there is considerably less coincidence; e.g., only 38% of the pyrolysis products of desert soil were found in the Murray meteorite, whereas there was a 36% correlation between M. luteus and Murray and only 30% of the pyrolysis products of B. subtilis were in common with those of Fig Tree Shale. There is, therefore, a clear distinction between the pyrolysates of contemporary biological organic matter and those of meteoritic or fossil organics.

If the organic analysis of the Martian surface by Py-GC-MS yields pyrolysis fragments which are indicative of complex macromolecular structures such as proteins and carbohydrates, this will provide strong presumptive evidence of life. Such an analysis would be particularly conclusive if one or more of the direct biology experiments also gives a positive result. In addition, the present work clearly demonstrates that the type of pyrolysis data obtained from biologically active soils is entirely consistent with a microbial form of life.

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