Pyrolysis-Spectrometry: Automated Identification of Microbes

The search for life on other planets spotlighted the need for automated methods for identification of microorganisms. On a distant planet, there is no technician to take three or more days to grow sufficient quantities of an organism and subject it to a battery of physical and biochemical tests that require subjective evaluation of results. It was necessary to develop tests that rely strictly on chemistry and spectrometry and that, above all, produce reliable and unequivocal results. Once the fundamentals of these new techniques were developed, their applications on Earth were quickly recognized and soon overshadowed the more esoteric needs of the space program.

Today, several automated and semiautomated techniques promise to reduce the time required for identification of microorganisms to less then a day and to make the ultimate identification more accurate. Perhaps even more important, the techniques may provide new tools for examination of the structure and chemistry of many types of cells.

GC, MS Most Common

The two most widely studied techniques are combinations of pyrolysis and either gas-liquid chromatography (GC) or mass spectrometry (MS). In these techniques, the organic sample is heated very quickly—usually in less than 1 second—to a temperature between 300° and 800°C in the absence of air. As the compound is degraded by the heat, fragments from proteins, carbohydrates, lipids, nucleic acids, and other molecules are released and analyzed by either GC or MS. The third and newest technique is called linear programmed thermal degradation mass spectroscopy, or LPTD-MS. In this technique, the sample is heated to about 400°C at a slow, predetermined rate in the absence of air, and the products are analyzed by MS. This process is thought to allow the decomposition products to be analyzed sequentially rather than all at once.

The concept of identifying microorganisms from pyrolysis fragments was probably first proposed about 1961 by Vance I. Oyama of the National Aeronautics and Space Administration's Ames Research Center, who suggested that it would be a good way to look for microorganisms on the moon. One of the first people to study the technique extensively was Eugene Reiner of the Public Health Service's Center for Disease Control in Atlanta. In 1962, he began pyrolyzing bacterial and mammalian cells and analyzing the products by GC. He found that the technique produces a pyrogram or chemical fingerprint that is apparently peculiar to each species of microorganism and that can be used to distinguish between even closely related strains. The technique was very slow to gain acceptance, however. Because of deficiencies in the equipment available during the 1960's, many investigators were unable to duplicate Reiner's results, became discouraged, and dropped out of the field.

With the advent of better equipment, Reiner and others have been able to study a wide variety of pathogenic and nonpathogenic microorganisms. Reiner has now shown that the technique can be used to provide unequivocal identification of these organisms if reference pyrograms are available. Similar results have been achieved by Henk L. C. Meuzelaar and his associates at the Institute for Atomic and Molecular Physics in Amsterdam, the Netherlands, and by Gordon S. Oxborrow, Norman D. Fields, and John R. Puleo at the Jet Propulsion Laboratory's Planetary Quarantine Laboratory at the Kennedy Space Center in Florida.

All the investigators report that the technique is very sensitive. Reiner says, for example, that it can distinguish between bacterial strains that differ by only one gene product. By way of illustration, he cites experiments in which he has distinguished between bacteria that differ only in the kind of protein found in their flagella. Puleo and his associates have shown that the technique can be used to distinguish between various spore-forming *Bacillus* species and can even indicate when cultured *Bacillus* are ready to undergo sporulation.

Reiner has found that the technique is useful for studying other types of cells also. He has shown, for example, that it can distinguish among mammalian cells from different parts of the body. In preliminary experiments, he has also shown that it can distinguish between malignant and healthy cells and between healthy cells and cells with genetic errors of metabolism. He has particularly high hopes for the latter capability and argues that it could greatly reduce the time required to identify genetic defects in fetal cells obtained by amniocentesis.

No investigators are yet using pyrolysis-GC routinely because of several problems. One problem common to all the techniques is the necessity for standardization of procedures for growing and harvesting the microorganisms and obtaining pyrograms. (For most applications, microorganisms must be cultured for at least 12 hours to obtain sufficient quantities for analysis.) For some organisms, such as species of *Bacillus*, Puleo and his associates have shown that slightly different pyrograms are obtained when the organisms are grown on different media, when they are harvested by different techniques, and when they are prepared for analysis in different ways. This is not true for all organisms; both Reiner and Meuzelaar have shown that the conditions of sample preparation make almost no difference in pyrograms obtained with *Mycobacteria*.

Pyrolyzers a Problem

Another major source of variation is the pyrolyzer itself. Different pyrograms can often be obtained for the same organism if different pyrolyzers are used. Most investigators now agree that sample preparation methods should be standardized in all laboratories if there is to be comparability of results among investigators; some also feel that all laboratories should use the same pyrolyzers. Oxborrow, Fields, and Puleo have developed a sample preparation technique that they feel minimizes analytical differences that arise from differences in sample preparation and they suggest that it would be a good candidate for a universal method.

A second shared problem is the need for a large catalog of sample pyrograms with which to compare a pyrogram of an unknown specimen. This problem is related to the first one because a catalog of pyrograms obtained in one laboratory may be of little use to someone from another laboratory who uses a different method of sample preparation and a different pyrolyzer and GC column. Some investigators are nonetheless preparing catalogs for their own purposes. Fields and Puleo, for example, have begun a catalog of spore-forming *Bacillus* species that might escape sterilization procedures. Reiner has already developed a catalog of *Salmonella* species that are

most likely to be encountered at the Center for Disease Control, and he would like to develop a catalog of other pathogenic organisms. Both groups hope that pyrolysis-GC will be used routinely if a large number of catalogs are available.

Other problems are specifically associated with the use of GC. Most GC columns, for example, are not capable of separating all the components produced by pyrolysis—although some high-resolution columns can separate as many as 200 products. Columns also undergo a gradual deterioration with extended use and frequently a rather sharp difference

Speaking of Science

An Illuminating New Use for Solar Energy

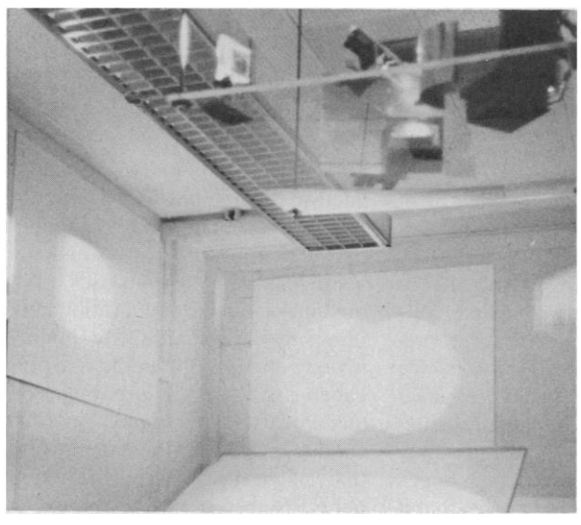
Solar-powered alternatives have been suggested as supplements or replacements for a host of traditional items—hot water heaters, furnaces, air-conditioners, electric home generators, and even central power stations. The rate at which this list has been lengthening should have been a warning that no piece of traditional technology was secure against the solar onslaught. But who would have suspected that inventors of solar-devices would propose a better way of illuminating a room than using a light bulb?

Two researchers working at a government laboratory in the southwest have conducted tests and made projections which suggest that indoor lighting is a sensible application of solar energy. In fact, they say it may be one of the more economic applications, because "the dollar value of sunlight used as light is about 10 times its dollar value when used as heat."

Put a sun-tracking mirror on the roof of a building, reflect the light onto a fixed spherical mirror, beam it into the building through a small glass porthole set in the roof, project it into the interior rooms of the building that lack windows, and the result is a solar lighting system.

In the few instances in which it has been tried, the effect is reportedly quite pleasing esthetically, and when clouds pass overhead they can be seen running across the image of the sun projected into the solar-lit room. Such a system could serve as a local weather indicator, providing a measure of psychological relief in office buildings, and could also have other benefits. If one system were used to illuminate many rooms on different floors, it could create new possibilities for interoffice communication as light beams destined for lower floors pass through the offices above.

One of the first installations of such a system for lighting purposes was at the Hyatt Regency Hotel in Chicago in 1974, where three beams were used to create a novel effect on the glassed roof of the hotel lobby. More recently, two physicists have set up a solar indoor lighting system in an office at the Sandia Laboratories in Albuquerque, New Mexico. The Sandia adaptation was motivated as much as anything by the desire of the researchers. Michel Duguay and Robert Edgar, to improve the conditions in their shut-in offices. Solar lighting has since become a full-time avocation for Duguay.



To light the Sandia office, a small flat mirror with an area of 0.3 square meters collects sunlight all day by following the sun under the direction of an electronic control circuit. A variety of mirrors and lenses split the beam inside the building and transmit the sunlight wherever it is needed. The accompanying photograph shows one corner of the sunlit office. The two circles are images of the sun projected onto a piece of white cardboard, and under the circles is a broad patch of light produced by a third beam hitting a piece of white diffusing plastic. The third beam provides illumination for a desk underneath the diffuser. Under clear skies in Albuquerque, the system provides 205 watts of sunlight, which is reported by the physicists who have tried it to be "better than the 250 watts of fluorescent lighting" that had been previously installed in the 3- by 5-meter room.

An additional advantage of using focusing optics, the Sandia researchers report, is that filters can be inserted in the concentrated beams to separate out the infrared portion of the spectrum, which produces heat but no light. Under ideal conditions, they report, about four times less heat is produced than with fluorescent lamps. The infrared need not be thrown away, however. Duguay is quite enthusiastic about using it for producing electricity in a photovoltaic cell.

Because incandescent bulbs are only 10 percent efficient at producing light from electricity and fluorescent bulbs are only 20 percent efficient, solar lighting has an advantage over the other applications of solar energy. Duguay estimates that the value of the sunlight from a 1-square-meter collector that tracks the sun in Albuquerque is $12 per year if it is used for heating or producing electricity in systems that are available today and $120 per year if it is used for lighting.

Solar lighting is not for every situation. It would seem to have limited applicability in homes, which have few interior rooms. And office buildings, which tend to have more windowless rooms, could alternatively be designed with skylights, interior courts, and light wells. Perhaps the idea is most applicable to existing buildings, but even then occupants would want electric lighting systems as well. For there is no doubt that when the sun goes down, a solar lighting system is sure to go out.—WILLIAM D. METZ

in the pyrograms is obtained when an old column is replaced with a new one. Chemists have overcome these problems pretty much by using relative retention times, but microbiologists do not seem to have adopted this approach yet. A more serious problem is that of putting data from the GC detector into digital form for analysis by computers. Most conventional digital integrators are not able to cope with the large amount of data produced by pyrolysis, and investigators have generally had to compare pyrograms manually. A few investigators, such as Colin Gabridge and John Norris of the Meat Research Institute in Bristol, England, have begun to make progress in data processing with some techniques originally devised for pyrolysis-MS. It will, however, still be some time before automated data processing is used routinely with pyrolysis-GC. Considering these problems, many investigators argue that the best results can be achieved by analyzing pyrolysis products with MS.

The greatest amount of experience with pyrolysis-MS has been obtained by Meuzelaar, Piet G. Kistemaker, and their associates at the Institute for Atomic and Molecular Physics. Investigators in North America who have worked with the technique include Patricia A. Quinn of The Hospital for Sick Children in Toronto, Catherine Fenselau of the Johns Hopkins University School of Medicine, and John P. Anhalt of the Mayo Clinic. A life detector based on pyrolysis and a combination of GC and MS was also developed for the Viking missions to Mars by a team headed by Klaus Biemann of the Massachusetts Institute of Technology.

More Information from MS

Meuzelaar and Kistemaker use a data processor to obtain repeated spectrums of pyrolysis products during the course of the pyrolysis. These are then summed to produce a pyrogram that represents all of the components produced during pyrolysis. They found that, like GC, MS produces a unique pyrogram for each organism. More important, they found that the technique can separate nearly all the products produced during pyrolysis and, if a high-resolution spectrometer is used, it can identify the elemental composition of most of the molecules present. As an example of the capability of the technique, Meuzelaar and Kistemaker used pyrolysis-MS on the bacterium *Pseudomonas putida*. They found that there were more than 180 different ions with mass-to-charge ratios less than 300. From these, they were able to identify 66 different compounds. Some 60 of these were identical to compounds identified by Peter G. Simmonds of the University of Bristol in the pyrolysis-MS of *Bacillus subtilis*, indicating the similarities between even considerably different species of bacteria.

Meuzelaar and his associates, often in collaboration with investigators from other institutions, have used pyrolysis-MS to conduct preliminary studies on a wide variety of materials, including fungi, viruses, organic polymers, glucose polymers, proteins, and DNA. In each case, they have shown that the technique holds great promise for identifying materials and studying their structures. Because of the large number of samples that are submitted to them by investigators who wish to test the technique, Meuzelaar and Kistemaker have developed a highly automated instrument that can produce pyrograms for as many as 40 samples per hour. Unlike the case with GC, they have also found that bacterial colonies can be sampled directly from culture plates with no need for preparation of the sample.

Fenselau and Anhalt used pyrolysis-MS to examine several pathogenic species of bacteria. They pyrolyzed the organisms at 300° to 350°C—a much lower temperature than that used by other investigators—in hopes of finding larger molecular fragments. They found that the pyrograms obtained with gram-negative bacteria result primarily from pyrolysis products of phospholipids and ubiquinones, both of which are known to be present in substantial quantities in those microorganisms. Quinn is using pyrolysis-MS to examine *Mycoplasma*, a very small organism that is considered to be intermediate between bacteria and viruses. And Biemann and his associates have demonstrated that the device aboard the Viking lander can detect and identify very small quantities of organic materials that might have been synthesized by living organisms.

The principal advantages of pyrolysis-MS are the wealth of detailed information provided by the pyrograms and the speed with which the pyrograms are produced; Meuzelaar's instrument can handle 40 samples per hour, whereas a good gas chromatograph can handle no more than two. The major disadvantages are cost and availability. Mass spectrometers can cost five to ten times as much as gas chromatographs. A gas chromatograph might thus be within the reach of clinical laboratories in almost every hospital, for example, whereas a mass spectrometer might be available only on a shared or regional basis. Reiner also argues that the reproducibility of MS is not as good as that obtained with GC—largely because the ionization sources tend to become contaminated—and that mass spectrometers are less stable and more susceptible to breakdowns. Meuzelaar, however, seems to have overcome these problems satisfactorily.

A variant of pyrolysis-MS is LPTD-MS, which has been developed by Terence H. Risby of the Pennsylvania State University and Alfred L. Yergey of Scientific Instruments Research Corporation in Baltimore. In this technique, the temperature of the sample is raised to 400°C at a rate of 20°C per minute; mass spectrums are obtained throughout the course of the heating. Yergey and Risby argue that the results obtained with their technique are just as reproducible as those obtained with conventional pyrolysis-MS, but that a great deal more information is contained in the pyrograms. They, like Anhalt and Fenselau, think that the slower decomposition of the sample yields larger fragments of the cell or macromolecule and thus provides more structural information. Some investigators argue, however, that the slow heating cooks the sample and leads to the production of molecular species that would not be there otherwise.

Pyrograms from Malignant Cells

Like the other investigators, Yergey and Risby have shown that their technique apparently produces pyrograms that are characteristic of each microorganism. They have also shown, working with Harvey M. Golomb of the University of Chicago Hospitals, that the patterns produced by healthy human cells are different from those produced by malignant cells and that cells from patients with the same form of malignancy yield similar patterns. Yergey thus suggests that LPTD-MS might be useful for the characterization of leukemias and other types of blood diseases.

Because of the magnitude of the data that must be processed, LPTD-MS may never be used for routine identification of microorganisms. It is, however, a promising tool for investigating chemical and biological structures. Golomb, Yergey, and Risby are currently beginning studies, for example, to try to identify the sources of the differences in pyrograms of leukemic lymphocytes. To this end, they are extracting lipids and lipoproteins from leukemic cells and their subcellular fractions and analyzing them with the same technique. In this way, they hope to identify specific biochemical differences between cells from differ-

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ent types of leukemias and between leukemic cells and healthy ones. Identification of these differences could, of course, provide a great deal of information about the nature of cancer.

Yergey predicts that the technique will also be useful for investigating other materials. He and Risby have used it, for instance, to study the depolymerization of polyisobutylene. They find that the polymeric bonds break in a reproducible fashion during the heating and that the spectral and thermal data can be used to calculate the thermodynamics of depolymerization. The values they have obtained in this manner appear reasonable when compared with data from prior studies with related compounds, Yergey says, but there are no available data for polyisobutylene to compare them with.

The principal limitation of LPTD-MS in studying such problems is the low final temperature that is now achieved in the pyrolysis. Many of the organic and biopolymers he would like to examine, Yergey says, do not decompose appreciably at temperatures less than 400°C. Yergey and Risby are thus working to increase substantially the temperatures that can be achieved by their probe. They are also working on the data processing problem to ensure that all of the available data that is collected is used.

There are, of course, many other potential applications where all of the techniques may prove valuable. In particular, a great deal more work will certainly be done in the study of yeasts, molds, fungi, and viruses. It might even be possible to use them for the study of intact insects, as has already been attempted with cockroaches. But it will be many years before such applications are routine. The reliability of the techniques will have to be firmly established before they will be trusted by most microbiologists, who still prefer the older, better-known biochemical and serological tests. Some investigators also suggest that it will be necessary to overcome the reluctance of microbiologists toward spending large sums of money for instrumentation.

Nonetheless, many investigators have already conducted preliminary studies, and these show that the techniques have great potential; larger bodies of data are now being collected to confirm that potential. The techniques have already cleared some major hurdles of instrumentation, Quinn says, and, once their potential and reliability are confirmed, acceptance will not be far behind.—THOMAS H. MAUGH II

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