Stereoisomeric Specificity and Soil Gas Disequilibria: Implications for Martian Life Detection

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Variations in the atmospheric composition of soil samples were monitored by mass spectrometry after the addition of mixtures of D- and L-carbohydrates and/or amino acids. The changes in concentrations of CO₂ in these experiments were found to be related to the stereoisomeric configurations of the compounds with which the soil samples were enriched. The potential of this relationship provides a comparatively simple approach for detecting life in extraterrestrial soils.

Biological activity of a living entity in an enclosed environment tends to result in a variation in the atmospheric constituents (7). The biological processes in soil which result in chemical interactions with the atmosphere (gas disequilibria, 15) are widely known and well documented (10). Monitoring the variations in the concentrations of soil gases (CO₂, N₂, NO, H₂, CH₄, O₂, etc.) with a mass spectrometer has been proposed as a unified approach to Martian life detection (12). Stereoisomeric specificity is considered a unique feature of living systems and is in itself generally accepted as evidence for life (4, 6, 9). Most enzymatically catalyzed reactions exhibit specificity for one of the enantiomers of a given stereoisomeric organic compound. The application of this property has been proposed as a potential life detection technique (5). Absolute specificity, however, is not always observed, and the exceptions which have been discovered preclude simple generalization. Amino acid racemases have been reported for many common species of bacteria, and a variety of enzymes are known to act on both the D and L forms of certain carbohydrates, although it is a rare exception when these enzymes act with equal rates (1).

H. C. Urey (1969, personal communication) proposed that extraterrestrial life detection experiments would be more definitive if differences in the evolution and types of gases, measured with a mass spectrometer, were associated with the stereoisomeric forms of organic compounds added to soil samples. Following the suggestion of Urey, experiments have been conducted to determine whether there is a specific response by untreated soils to laboratory enrichment with the D or L isomers of carbohydrates and amino acids.

MATERIALS AND METHODS

Organic enrichments. Mixtures of five carbohydrates (glucose, arabinose, fucose, mannose, and xylose) and mixtures of seven amino acids (serine, valine, leucine, glutamic acid, methionine, phenylalanine, and tryptophan) were added to soil samples in replicate experiments. Aqueous solutions were prepared containing a total of 50 μM of equimolar combinations of the compounds per ml in either the D or L form. The solutions were sterilized by passage through membrane filters (Millipore Corp.) of 22-μm porosity.

Soils. Samples consisted of three mixtures of intentionally uncharacterized soil from Tucson, Ariz., which were prepared from cultivated and non-cultivated, surface and subsurface sources to assure a heterogeneous biological activity and composition. These three mixtures, which were collected during different seasons, were not completely air dried when studied. Additional soils included an Antarctic sample (2) and four characterized Arizona soils (3): one sample of Pima clay loam (Cumulic Haplustolls, Fine-silty, mixed, thermic) which was obtained from a depth of 91 to 97 cm at Silver Bell, Ariz.; one cultivated Rillito loam (Typic Calcic Haplustolls, Coarse-loamy, mixed, hyperthermic) from Marana, Ariz.; and two sandy loams (Typic Hapludands, Coarse-loamy, mixed, thermic) collected from the surface (0 to 7 cm) at Sonora, Ariz. The Antarctic soil had been stored at −60°C in sealed containers. The other characterized soils had been lyophilized and stored at room temperature and atmospheric pressure in sealed bulk containers.

Gas measurements. In a given experiment, 1 ml of appropriate stock solution (see above) was added to a 1-g sample of screened soil (18- by 14-mesh screen) previously equilibrated with a nitrogen or argon
atmosphere. The samples were contained in 10-ml gas-tight vials fitted with glass tubing and vacuum sampling stopcocks. They were incubated at room temperature. Duplicate vials were prepared containing the respective untreated soils, which had been autoclaved for 2 h at 121 C in shallow pans immediately prior to each experiment. Additional control vials contained the respective untreated soils, to which only 1 ml of sterile distilled water was added. After 1 h and at scheduled intervals thereafter, duplicate samples of approximately 10 µl of gas were directly transferred through evacuated connecting glass tubing into the gas inlet system of a Hitachi RMU-6E mass spectrometer. Low resolution, 3-s mass scans were obtained between m/e = 12 and m/e = 50. The ionization voltage was 70 eV, the entrance and exit slit widths of the magnetic sector were set at 200 µm, the electron multiplier sensitivity was 1x, and the multiplier voltage was 1,300 V. All mass spectral peak intensities were corrected for instrument background. Ion statistical errors of CO₃₃ peak heights calculated as coefficients of variation were found to be 5.9%. High resolution, low-scanning-speed spectra were used periodically to ensure that m/e = 44 consisted of only one component, i.e., CO₂.

Data processing and analyses. Altogether more than 19,000 measurements of mass spectrometric ion intensities were analyzed in ten experiments. All computations were performed with the CDC 6400 computer at The University of Arizona Computer Center. Depending on the experimental atmosphere (N₂ or Ar), the carbon dioxide-nitrogen intensity ratios (m/e = 44/14 and 44/28) or CO₂-Ar ratios (m/e = 44/40) were expressed as log₁₀ for statistical analyses. The log ratios for each experiment were analyzed according to a model I three-way factorial analysis of variance design (14) where type of soil enrichment, heat treatment, and time were assigned as factors. The residual or error mean square (variation not attributed to the factors singly or to their first- and second-order interactions) was regarded as the best estimate of the random variability. The 99% confidence limits (CL₉₉) for each mean of n measurements from replicated vials at a given time were computed as follows:

\[ CL_{99} = \text{mean log ratio } \pm (t) \sqrt{\frac{\text{error mean square}}{n}} \]

where t is Student's t value for 0.01 probability of type I error for the corresponding degrees of freedom associated with the error mean square.

RESULTS

In preliminary experiments mixtures of the five carbohdrates or the seven amino acids in both D or L forms were added to a freshly prepared mixture of Tucson soils and sampled on the initial day and every 3rd day thereafter until the 15th day. CO₂-N₂ ratios of samples treated with the seven amino acids in the D or L form were significantly different on the 3rd, 6th, 9th, and 12th days of sampling. Treatment of soils with the L isomers resulted in the production of higher CO₂ concentrations, with the exception of the 12th day, on which a much higher CO₂ concentration was associated with the D isomers (Table 1). Samples treated with the mixture of the five carbohydrates showed significantly higher CO₂ content on the 3rd, 9th, and 12th days when the D isomers were added (Table 1). Concentrations of CO₂ were not significantly different on the 6th and 15th days. Combinations of D- or L-carbohydrate mixtures with D- or L-amino acid mixtures were also studied. Addition of a mixture of the five carbohydrates in either the D or L form to the D form of the seven combined amino acids resulted in significant differences in gas ratios on the 3rd and 12th days (Table 1). Addition of the D- or L-carbohydrate mixtures to the L forms of the seven amino acids resulted in less pronounced differences, but differences in gas ratios on the 3rd, 6th, 9th, 12th, and 15th days were noted (differences obtained on the 3rd and 15th days were statistically significant, i.e., P < 0.01; Table 1). As was determined mass spectrometrically, the gas ratios of unheated soil to which only distilled water had been added did not differ in a statistically significant manner from the gas ratios observed in the autoclaved soil samples. These latter CO₂-N₂ ratios were essentially constant throughout an incubation period which extended to 36 days.

In a second experiment mixtures of the same carbohydrates and amino acids were added in either the D or L forms to another composite mixture of soil samples from Tucson. Significantly different gas ratios were again associated with the addition of the isomeric forms of these compounds (Fig. 1). A greater response expressed as CO₂-N₂ ratios was associated with the mixture of L-amino acids (Fig. 1a) and the D-carbohydrates (Fig. 1b). Instances in which the 99% confidence limits do not overlap in these illustrations can be interpreted as significant differences between positions of respective means. In this experiment, a statistically significant coincident, transient decrease in gas ratios was observed in vials containing either the D or L isomers on the 5th day.

Similar experiments have been performed utilizing nine different soil samples ranging from fertile to agriculturally barren soils. Addition of mixtures of the same five carbohydrates or seven amino acids in either the D or L forms has always resulted in a differential CO₂ evolution in at least one observation during incubation. The magnitudes of gas ratios obtained throughout the incubation were never the same for different soils, as is illustrated in Fig. 2,
which compares the responses obtained after the addition of \( \text{D} \) or \( \text{L} \) isomers of the five carbohydrates to two different soils in argon atmospheres. Samples of the subsurface Pima Clay loam responded similarly to either the \( \text{D} \) or \( \text{L} \) forms of carbohydrates during the first 9 days.

### Table 1. Relative CO\(_2\) content of gas in vials containing mixed Tucson soils in a \( \text{N}_2 \) atmosphere after enrichment with either \( \text{D} \) or \( \text{L} \) isomers of seven amino acids and five carbohydrates (CHO)\(^a\)

| Incubation period (days) | Amino acids | Carbohydrates | \( \text{d}-\text{amino acids plus:} \) | \( \text{l}-\text{amino acids plus:} \) | Water controls |
|-------------------------|-------------|--------------|-------------------------------|----------------|---------------|
|                         | \( \text{D-isomers} \) | \( \text{L-isomers} \) | \( \text{D-isomers} \) | \( \text{L-isomers} \) | \( \text{D-(CHO)} \) | \( \text{L-(CHO)} \) | \( \text{D-(CHO)} \) | \( \text{L-(CHO)} \) | \( \text{Water controls} \) |
| 0                       | 0.018       | 0.022        | 0.012                         | 0.013          | 0.011         | 0.019         | 0.022         | 0.014         | 0.010         |
| 3                       | 0.026       | 0.156        | 0.555                         | 0.212          | 0.054         | 0.322         | 0.187         | 0.326         | 0.209         |
| 6                       | 0.076       | 0.214        | 0.361                         | 0.292          | 0.267         | 0.311         | 0.267         | 0.350         | 0.047         |
| 9                       | 0.096       | 0.344        | 0.453                         | 0.232          | 0.226         | 0.207         | 0.283         | 0.340         | 0.052         |
| 12                      | 0.782       | 0.213        | 0.312                         | 0.147          | 0.166         | 0.632         | 0.221         | 0.261         | 0.113         |
| 15                      | 0.113       | 0.138        | 0.303                         | 0.303          | 0.182         | 0.212         | 0.152         | 0.334         | 0.044         |

\(^a\)Values are mean log ratios of \( \text{m/e} = 44 - \text{m/e} = 14 \) ion intensities; 99% confidence limits = \( \pm 0.046 \).

**Fig. 1.** Gas disequilibrium in samples of Tucson, Ariz. soil mixtures during incubation in \( \text{N}_2 \) atmosphere at room temperature after addition of (a) isomers of a mixture of seven amino acids and (b) isomers of a mixture of five carbohydrates. Symbols: \( \text{■, D isomers; ○, L isomers; ▲, addition of water only. Vertical lines represent the 99% confidence limits of each mean ratio of experiments with duplicate samples. Vertical axis shows the log ratios of ion intensities.} \)

**Fig. 2.** Gas disequilibrium in (a) clay loam soil (Silver Bell, Arizona; 91 to 97 cm depth) and (b) Antarctic dry valley soil (no. 500) during incubation in \( \text{Ar} \) atmosphere at room temperature after addition of isomers of a mixture of five carbohydrates. Symbols: \( \text{■, D isomers; ○, L isomers; ▲, water controls. Vertical lines represent the 99% confidence limits of each mean ratio of experiments with triplicate samples with the carbohydrates and duplicate samples with the control. Vertical axis shows the log ratios of ion intensities.} \)
of incubation. Thereafter, significantly higher CO₂-Ar ratios were associated with the D isomers (Fig. 2a). Much less change in CO₂-Ar ratios was observed for samples of Antarctic soil treated similarly, even though there must have been substantial proliferation during the incubation period. The gas ratios for samples treated with the D or L forms were statistically different on the 8th day and later (Fig. 2b).

**DISCUSSION**

Two aspects of these experiments merit discussion: (i) the application of mass spectrometry to the study of stereoisomeric specificity of soil gas disequilibria and (ii) the application of this technique to possible Martian life detection.

Direct analysis by mass spectrometry was chosen over conventional manometric or gravimetric procedures because the sensitivity of this instrumentation permits repeated analyses of minute quantities (<10 μl) of gas from small samples of soil. Moreover, it permits the simultaneous analysis of the total gas composition, which could conceivably include H₂S, CH₄, H₂, NO, NO₂, NH₃, O₂, CO₂, N₂, etc. In our experiments, however, only CO₂ concentrations were observed to change significantly. However, in 1-g control samples of soil to which only distilled water had been added, the biological activity was insufficient to produce a detectable change in the CO₂ concentrations within 15 days. This observation agrees with the general concept that the activity of the soil biota is limited by available nutritional sources (13).

Biological experiments have been included in the planned Viking lander for detecting possible metabolic activity in Martian soil samples (8). The experiments are designed for detection of the following phenomena: (i) incorporation of reference ¹⁴CO₂ and ¹²CO into possible Martian organic compounds, (ii) release of ¹⁴CO₂ by Martian processes from reference terrestrial organic compounds labeled with ¹³C, and (iii) exchange of gases resulting from reactions between Martian soil and terrestrial organic and inorganic compounds. The terrestrial stereoisomeric organic constituents included in the latter experiments will be furnished in racemic mixtures.

However, distinguishing between the stereoisomeric forms of organic compounds in automated extraterrestrial life detection experiments or in returned Martian soil samples (16) would reduce the ambiguity of the quantitative measurements (the response signal). This signal must in turn be interpreted as positive or negative evidence for the presence of life. Expectations are that a false-positive signal would be less likely than a false-negative signal. For example, changes in gas ratios involving CO₂ might be attributable to changes in soil pH which affect the solubility of CO₂ in soil constituents. If the resultant positive response signal from such an effect could be evaluated on the basis of the stereoisomeric forms of the reference compounds, the information acquired would allow a less ambiguous interpretation of the response signal.

The exploitation of stereoisomeric specificity in a life detection system has previously been proposed by Halpern et al. (5). Their approach is based on the addition of racemic mixtures of amino acids to soil. After incubation the unaltered amino acids are extracted and assayed for relative concentrations of their enantiomers. Optically active reagents are required to produce diastereoisomeric derivatives which can be subsequently resolved by gas chromatography and mass spectrometry. A particular merit of their approach is the ability to identify the specific isomer which was acted upon by processes in the soil, but the experimental complexity of this procedure is a disadvantage. A second possible disadvantage derives from the fact that some organic compounds can exert a profound stimulatory or inhibitory effect upon a biological system without undergoing a measurable incorporation or decomposition, for example, antibiotics, vitamins, and auxins (7). In contrast to the approach of Halpern et al. (6), our technique does not specify how the added compounds are utilized or the molecular sources of the gas constituents which undergo changes in concentration.

The approach taken in the present study relates only soil gas disequilibrium responses to the isomeric forms of the organic enrichments. The compounds used were selected because of their biological significance and the ready availability of both isomeric forms in highly purified preparation. Nevertheless, a wide variety of other stereoisomeric compounds could be included in mixtures, and the net response of the living system could be monitored without the necessity of measuring the extent of substrate decomposition. The use of compounds labeled with stable isotopes would be an additional aid in ascertaining the origin of the evolved gases.

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LITERATURE CITED

1. Bentley, R. 1969. Molecular asymmetry in biology, vol. I. Academic Press Inc., New York.
2. Cameron, R. E., and E. L. Merek. 1971. Growth of bacteria in soils from Antarctic dry valleys. Technical report no. 32-1522, NASA. Jet Propulsion Lab., California Institute of Technology, Pasadena.
3. Gelderman, F. W. 1972. Soil survey of the Tucson-Avra Valley area, Arizona. Soil Conservation Service, U.S. Department of Agriculture, Washington, D.C.
4. Halpern, B., J. W. Westley, P. J. Anderson, and J. Lederberg. 1966. Demonstration of the stereospecific action of microorganisms in soil by gas liquid chromatography. Anal. Biochem. 17:179-182.
5. Halpern, B., J. W. Westley, E. C. Levinthal, and J. Lederberg. 1967. The Pasteur probe: an assay for molecular asymmetry, p. 239-249. In A. H. Brown and F. G. Favorite (ed.), Life sciences and space research. A symposium. North-Holland Publishing Co., Amsterdam.
6. Halpern, B., J. W. Westley, I. von Wredenhagen, and J. Lederberg. 1966. Optical resolution of D, L amino acids by gas chromatography and mass spectrometry. Biochem. Biophys. Res. Commun. 20:710-714.
7. Hitchcock, D. R., and J. E. Lovelock. 1967. Life detection by atmospheric analysis. Icarus 7:149-159.
8. Klein, H. P., J. Lederberg, and A. Rich. 1972. Biological experiments: the Viking Mars lander. Icarus 16:139-146.
9. Lederberg, J. 1966. Signs of life. Criterion-system of exobiology. Nature (London) 207:9-13.
10. McLaren, A. D., and G. H. Peterson. 1967. Soil biochemistry. Marcel Dekker, Inc., New York.
11. Oyama, V. I. 1972. The gas exchange experiment for life detection: the Viking Mars lander. Icarus 16:167-184.
12. Radmer, R., and B. Kok. 1971. A unified procedure for the detection of life on Mars. Science 174:233-239.
13. Russell, E. W. 1973. Soil conditions and plant growth, p. 221, 10th ed. Longmans Green and Co., New York.
14. Sokal, R. R., and F. J. Rohlf. 1969. Biometry, 1st ed. W. H. Freeman and Co., San Francisco.
15. Tiedje, J. M., S. H. Lai, and A. E. Erickson. 1973. Martian life detection based on "in situ" gas analysis. In T. Rosswall (ed.), Modern methods in the study of microbial ecology. Bull. Ecol. Res. Commun. (Stockholm) 17:489-497.
16. Young, R. S., and D. L. DeVincenzi. 1974. From Mars with love. Science 186:495-501.