Rate-Temperature Curves as an Unambiguous Indicator of Biological Activity in Soil

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Experiments are described in which we used a mass spectrometer to monitor O2 uptake of enclosed soil samples as a function of temperature. We found that an Arrhenius plot of the rate of O2 uptake showed pronounced local maxima attributable to biological activity, whereas similar plots of rates obtained with abiotic soils yielded straight lines. This procedure thus provides a basis for distinguishing biological from chemical activity for reactions, such as O2 uptake, that can occur via either biological or chemical pathways.

A basic problem encountered in experiments in which biological reaction (often occurring at a low rate) is monitored in a chemically active milieu (e.g., soil) is to distinguish biological from chemical activity. Over the temperature range compatible with biological processes, such biological activity will be superimposed on a chemical activity; i.e., at any given temperature the rate of a reaction (e.g., O2 uptake) will be the sum of the biological and non-biological rates. Attempts to separate these two activities by the use of a parallel sterilized sample are often ineffective, since sterilization usually alters the sample so that it no longer serves as an adequate control (8).

One way by which biological and chemical activities might be distinguished is by the use of rate-versus-temperature curves (7-9). Biological reactions show a temperature optimum and do not occur at elevated temperatures. Chemical reactions increase exponentially with temperature well beyond the range of biological reactions, so that a plot of the logarithm of the rate versus the inverse of the absolute temperature (an Arrhenius plot) describes a straight line.

Several years ago, we proposed and tested a control procedure for life detection experiments in which rates (e.g., rate of O2 uptake) measured at high, non-biological temperatures were used to infer the rates of non-biological reactions at lower, biological temperatures by the use of an Arrhenius plot (7, 8). The results of many experiments of this type suggested to us that the Arrhenius curve could be a self-sufficient and unambiguous indicator of biological activity. This suggestion is confirmed by the experiments reported in this paper. We observed that an Arrhenius plot of soil-mediated O2 uptake has a pronounced local maximum attributable to biological activity. Thus, the relative rates of these two reactions can be inferred, and an unambiguous conclusion for or against the presence of life can be made on the basis of this information.

MATERIALS AND METHODS

Early experiments were performed by methods similar to those described previously (9). In these experiments each soil sample was incubated in a Kimax glass tube (6 by 50 mm) provided with a Nupro SS4SA valve. The valves were used to close the vessels as well as to provide a molecular leak to the mass spectrometer. Different incubation temperatures were obtained by storing the valve-vessel assemblies in ovens of different temperatures. This technique gave satisfactory results, but allowed only a limited number of analyses in any single day.

Later experiments employed a technique in which soils were incubated in 7-ml (nominal) screw-capped septum vials (Pierce Chemical Co., Rockford, Ill.). A number of vials, each containing a soil sample, were stored at different temperatures. Enclosure and sampling ports were provided by Mininert valves (Pierce Chemical Co.) which allowed the sample to be withdrawn through a cylindrical gas chromatographic septum. This septum was replaced between measurements; we estimated that the error due to the introduction of atmospheric O2 to the reaction vial was less than 1%. When not being sampled, the contents of the vial were isolated from the external atmosphere by a Teflon valve. As a result, only glass and Teflon, and for short periods of time, silicone rubber, were in contact with the sample. At various time intervals (~30 min at the higher temperatures, about a week at low ones), small samples of gas were taken from the sample vials with a 100-μl gas syringe. The sample was injected through a gas chromatographic septum into an evacuated volume (~4 ml) that served as the mass spectrometer inlet system. This inlet volume was then sampled directly by using a Varian inlet valve.

Results obtained with the two techniques were essentially identical.

The mass spectrometer used for analysis was a modified residual gas analyzer (Consolidated Electro-
dynamics Corp. model 21-613). At least four repetitive measurements of m/e values 28 (N₂), 32 (O₂), 40 (Ar), and 44 (CO₂) were made of each gas sample. The Ar values were used as internal standards, and all measurements were normalized to this inert gas. The ratio of N₂ to Ar did not change significantly during these experiments.

The soil samples used in these studies were obtained from R. Johnson and E. Merek, Ames Research Center, Moffett Field, Calif. Most of these soils were originally acquired in 1967 and stored air-dried. These soils are quite well characterized, and some were used to test and standardize the biological experiments aboard the Mars Viking landers. An artificial soil (sand-carbon) was prepared by thoroughly mixing 1.3 g of decolorizing carbon into 200 g of acid-washed sand. This soil was intended to serve as an analog of Antarctic soil 542, an abiotic soil which has finely divided anthracite coal as its main carbon constituent (5).

RESULTS

Figure 1 shows the O₂ uptake kinetics observed when a moistened soil was incubated in a closed vessel at 28, 63, and 120°C. These three curves are typical examples of the kinetics observed at normal biological temperatures, elevated biological temperatures (~45 to 70°C), and temperatures high enough to inhibit most biological activity (>80°C). Note that as the incubation temperature increased, the linear time course was replaced by a time course with a pronounced lag, so that at early incubation times the O₂ uptake at 28°C was greater than that at 63°C. After this lag, however, the 63°C sample showed a rapid O₂ uptake, so that the integrated uptake rapidly overtook that observed at 28°C. At high non-biological temperatures (120°C) a rapid O₂ uptake without any appreciable lag was observed.

Figure 2 is an Arrhenius plot (i.e., ln k versus 1/T) of the complete series of experiments, of which the curves of Fig. 1 are representative samples. Each datum point was obtained graphically from the time course of O₂ uptake. The time span of individual experiments ranged from a few hours to many weeks, depending on the incubation temperature. (The observations at 28°C shown in Fig. 1 are the first datum points obtained in an experiment in which the sample was monitored for over 50 h.)

![Figure 2. Arrhenius plot of the rates of O₂ uptake at different temperatures by Siskiyou soil. Conditions were as in Fig. 1. Rates were computed from time courses like those of Fig. 1. Symbols: ■, initial rates, the first point being taken within 15 min after the sample was placed in the oven; □, rates computed from the rapid O₂ uptake observed after the lag (cf. Fig. 1, line B). See text for details. In this figure and in Fig. 3 the natural log of the rate (in micromoles of O₂ taken up per gram of soil-hour) is given on the left ordinate, and the absolute rate is given on the right ordinate.](http://aem.asm.org/)

![Figure 1. Time course of O₂ uptake by enclosed samples of Siskiyou soil incubated at 28°C (O), 63°C (Δ), and 120°C (□). A 7.75-g amount of soil and 1.11 ml of water were incubated in a total volume of 8.86 ml. This soil was a sandy loam with a pH (of the paste) of 6.3 and a bacterial content (assayed with 1% tryptic soy agar) of 5 x 10⁶ aerobes per g (2).](http://aem.asm.org/)
The solid squares of Fig. 2 represent rates obtained from the linear time courses observed at temperatures of \(<45\) and \(>80^\circ C\) (e.g., Fig. 1, lines A and C). In many cases (e.g., Fig. 1, line C) the time course was linear down to zero \(O_2\) concentration; in other cases the \(O_2\) uptake gradually decreased at low concentration, in which case the initial linear phase of the time course was used to calculate the rate. The pronounced linearity of the \(O_2\) uptake time course suggests that the rate is independent of \(O_2\) concentration down to a fairly low value. This apparent zero-order kinetic behavior is typical for many biological and chemical processes and points to a kinetic formulation of the following type: 
\[
d[O_2]/dt = (-k'E[O_2])/([O_2] + K_m),
\]
where \(k'\) is the rate constant, \(E\) is the concentration of active sites (catalysts), and \(K_m\) is the concentration at which the rate is half-maximal. This formalism is commonly used to describe the rate of enzyme-catalyzed reactions as well as heterogeneous chemical reactions (Michaelis-Menten and Langmuir mechanisms, respectively).

The apparent \(O_2\) half-saturation value for respiration by soil organisms is known to be quite low (\(<<1\% O_2\)), and thus one would expect the time course of biologically mediated \(O_2\) uptake to be linear to very low \(O_2\) tensions. It is more difficult to generalize in the case of non-biological \(O_2\) uptake, although our experience to date suggests that the \(K_m\) is also quite low (\(<1\%\)).

The open squares of Fig. 2 represent rates obtained from the descending slopes observed after the initial lag in \(O_2\) uptake observed in the temperature range between 45 and 70\(^\circ C\) (see Fig. 1, line B). We are clearly observing a biological process which is quite distinct from the one discussed above. Its possible nature is discussed below.

The lag in \(O_2\) uptake seen in this intermediate temperature range is peculiar and, to our knowledge, represents a new observation. This lag cannot be ascribed to slow temperature equilibration; using a thermocouple, we found that the soil temperature rapidly (in about 10 min) attained the temperature of the surrounding medium. In addition, the lag did not appear at higher temperatures, again suggesting that it was not due to limitations of temperature equilibration or gas transport.

The precision of our data does not warrant the calculation of the small rates during the lag. One would expect that \(O_2\) uptake should occur during the lag at a rate greater than or equal to the extrapolated chemical rate (Fig. 2, dashed line). However, the determination of rates of this magnitude requires incubation for many hours for measurable changes in \(O_2\) concentration to occur, by which time biological uptake has become significant. The descending dashed line extrapolation of the high-temperature data (Fig. 2, solid squares slope) depicts an estimate of the rate of the chemical processes at temperatures below 80\(^\circ C\). The rates observed during the lag phase are not far above these predicted values; i.e., the biological activity is minimal.

The intriguing \(O_2\) uptake kinetics and temperature profile observed in the intermediate temperature range probably reflect the process of spore activation during the lag, followed by germination (rapid \(O_2\) uptake). It has been recognized for some time that a moderate heat treatment, for example, 65\(^\circ C\) for 2 h, leads to the activation of dormant spores. (For a description of this topic, see references 4 and 10.) According to this interpretation the lag followed by a high rate of \(O_2\) uptake at intermediate temperatures is due to the rapid death of the indigenous mesophilic organisms and the activation and subsequent germination of another, more thermophilic flora. At first sight this large population of thermophilic soil flora is quite surprising. However, the temperature at the soil surface can be much higher (25 to 45\(^\circ C\)) than that of the surrounding air even in hot deserts (1), and thus temperatures comparable to our intermediate temperature range may be quite common in nature.

Figure 3 shows Arrhenius plots of the results obtained when the same procedure was applied to biologically inactive soils. Trace A was obtained by using an artificial soil made up of acid-washed sand and finely divided carbon, which displayed chemical activity but (presumably) little or no biological activity. The Arrhenius plot of these data describes a straight line; there is no local temperature maximum indicative of biological activity.

Similar results were obtained when a naturally occurring abiotic soil, Antarctic soil 542, was assayed (Fig. 3, trace B). Again, the Arrhenius plot describes a straight line, and there is no local maximum. This soil was very unreactive; the rates of \(O_2\) uptake observed at 62\(^\circ C\) were near the lower limit of our experimental technique.

The data in Fig. 3 confirm our thesis that an Arrhenius plot of the datum points obtained by using biologically inactive—but chemically active—soils should describe a straight line and that a local maximum, such as that shown in Fig. 2, is obtained exclusively with biologically active soils. Additionally, the \(O_2\) uptake kinetics obtained with these sterile soils were linear from the beginning of the incubation and showed no lag. This observation lends additional support
for the conclusion we reached above, that the lag kinetics reflects a biological process.

**DISCUSSION**

In a soil sample in which both chemical and biological mechanisms of O₂ uptake are occurring, the rate at any temperature can be expressed as the sum of the two processes. Figure 4 shows a set of theoretical curves, derived as explained in the figure legend, for different ratios of biological to chemical activity. Only a single biological process with an optimum of 37°C was considered. The biological activity was zero in curve E and increased in the sequence from curve D to curve A. The intermediate curves (Fig. 4, curves B through D) are of some interest, inasmuch as they provide a somewhat intuitive picture of the sensitivity limitations of this procedure.

As described above, at biological temperatures the effect of biological activity is to increase the rate (e.g., O₂ uptake rate) compared with what would be predicted on the basis of the extrapolated rate-temperature relationships for the chemical reactions. Thus, Fig. 4, curve A, predicts an increase attributable to biology of 25-

![Figure 3](http://aem.asm.org/)

**Fig. 3.** Arrhenius plot of the rates of O₂ uptake at different temperatures by an enclosed sample of sand-carbon artificial soil (see text) and Antarctic soil 542. A 8.04-g amount of sand-carbon (C) or a 6.63-g amount of Antarctic soil 542 (B) was incubated with 1.0 ml of water in a total volume of 8.86 ml.

![Figure 4](http://aem.asm.org/)

**Fig. 4.** Theoretical Arrhenius plots for different ratios of biological to chemical activity. Data were computed by the equation:

\[ v = C \cdot T \exp(-\Delta H^_/RT) \exp(\Delta S^_/R) \]

\[ + \frac{C_a T \exp(-\Delta H^a_/RT)}{1 + \exp(-\Delta H^a_/RT) \exp(\Delta S^a_/R)} \]

derived from the formulations of Eyring and co-workers (3, 6). In this equation \( v \) is the overall rate of uptake (e.g., of O₂) by a soil (equal to the sum of the biological and chemical rates); \( T \) is the absolute temperature; \( R \) is the gas constant; \( \Delta H^_/ \), \( \Delta H^a_/ \), and \( \Delta H_\) are the heats of activation for the chemical process, biological process, and thermal denaturation, respectively; \( \Delta S^_/ \) and \( \Delta S^a_/ \) are the entropies of the chemical process and thermal denaturation, respectively; \( C_a \) and \( C \) are constants lumping together \( k \) (the Boltzmann constant), \( h \) (Planck's constant), a concentration factor, and in the case of \( C_a \) the term exp(\( \Delta S^a_/R \)). Parameters for the soil biology reaction were taken from Johnson et al. (6): \( \Delta H^_/ = 20,000; \Delta H^a_/ = 13,400; \Delta H_ = 96,000; \Delta S_ = 306.14 \). Parameters for soil chemistry were selected such that the reaction would occur at a measurable rate at temperatures near 50°C. The constants \( C_a \) and \( C \) were chosen such that the biological contribution differed 10-fold between each of the adjacent curves A to D. Curve E had no biological component.
fold at 37°C, 80-fold at 20°C, and 120-fold at 10°C. At lower biology/chemistry ratios (curves C and D) this predicted increase is much less, and the interpretation (biology versus chemistry) correspondingly becomes more difficult.

Similar striking increases in the observed versus predicted rates can be derived from the actual data of Fig. 2. In this figure, the increase is about 45-fold at the biological temperature maximum (62°C) and 100-fold at 28°C. In contrast, Fig. 3 shows that no corresponding increase is apparent in abiotic soils.

The calculated curves of Fig. 4 reveal another convenient criterion for biological activity, viz a comparison of the different temperatures at which equal rates occur (rather than an extrapolation and comparison of rates at the same temperature, as described above). For example, in Fig. 4, curve A, the rate of biologically mediated O₂ uptake at 37°C was identical to the non-biological rate at 75°C, a "temperature decrement" of 38°C. (Note that both rates can be measured rather than obtained by extrapolation.) Similar large temperature decrements can be calculated from curve A at other (lower) temperatures. At lower ratios of biological to chemical activity, this decrement becomes smaller (it is only 13 to 15°C in curve B), until ultimately there is no "hump," i.e., no measured chemical rate corresponding to the measured, presumably biological rate (curves C and D). Under these circumstances the distinction between biological and chemical activity is less clear, since one must now distinguish biological versus non-biological activity on the basis of a break in the plot of ln k versus 1/T, assuming that the ΔHᵦ values for the two reactions are significantly different.

If we apply these same analyses to the data of Fig. 2, we again obtain large temperature decrements. For example, the O₂ uptake rate at the temperature optimum in Fig. 2 (62°C) corresponds to the non-biological rate at 100 to 105°C, a temperature decrement of ~40°C. A similar temperature decrement is observed at the rate corresponding to the local minimum at 80°C.

The data of Fig. 1 and 2 provide conclusive and unambiguous evidence of biological activity in a dried, rather poor soil that was not amended with any organic nutrient. Although we have measured complete Arrhenius curves in only a few soils, we have an abundance of three- and four-point data from a variety of soils. We found that even the poorer soils showed large (~100-fold) differences between the extrapolated chemical rate and the measured biological rate. The overall dynamic range of this type of measurement can be quite impressive. For example, we obtained conclusive evidence for biological activity in several soils acquired locally; the richest soil showed greater activity at 25°C than the poorest soil did at 90°C.

This life detection procedure should be applicable, in varying degrees, to several of the Mars missions currently envisioned. It would be especially suitable for studying a returned Mars soil sample, either on Earth or in Earth orbit. Although our experiments to date have focused almost exclusively on O₂ uptake reactions (i.e., respiration and combustion), this procedure could be fruitfully employed to determine the relative contributions of biological and chemical activity (at a given temperature) to any reaction.

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LITERATURE CITED


