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THE EFFECT OF STERILIZATION ON BIOLOGICAL, ORGANIC GEOCHEMICAL

AND MORPHOLOGICAL INFORMATION IN NATURAL SAMPLES

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A REPORT OF A STUDY BY LIFE SCIENCES, AMES RESEARCH CENTER,

TO THE PLANETARY BIOLOGY PROGRAM OFFICE, NASA, WASHINGTON, D.C.,

WITH REFERENCE TO A MARS SURFACE SAMPLE RETURN MISSION

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FOREWORD

The scientific results contained in this report shed some light on the loss
of biological, organic geochemical and morphological science information
that may occur should a Mars surface sample be sterilized prior to return
to Earth. The impetus for this work grew out of a symposium held at Ames
Research Center in October, 1973 to discuss various technical and scientific
aspects of a Mars sample return mission, with particular attention being
focused on the question of back contamination (see Appendix for symposium
proceedings). A major conclusion of this Symposium was that the most
desirable sample, retaining the maximum amount of scientific information,
would be one that was unaltered in any way. A second recommendation was
that more data was needed before a valid assessment could be made of the
magnitude of science loss should it be decided that a Mars sample had to
be sterilized prior to return. This report summarizes the results of
experimental studies carried out in the Life Sciences at Ames during the
last four months.

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INTRODUCTION

The potential opportunities for Mars Surface Sample Return (MSSR) Missions which exist in 1981, 1983/84 provide the scientific community with the possibilities of examining the organic geochemical and biological properties of the planet with a degree of detail not previously possible.

The limitations of planetary lander programs are traceable to restrictions arising from considerations such as spacecraft weight, power, and volume constraints. As a result, remote analyses are limited to "gross" analyses of surface samples in which a limited range of experiments are carried out at a fairly low level of resolution. In addition, such remote experiments possess little of the positive feed-back and resultant scenario changes which routinely take place in the usual laboratory experience. Thus exploration programs designed to investigate the chemical and biological properties of a planet by planetary landers are, by nature, self-limited in terms of the number of questions that can be asked. Failure to anticipate even the slightest environmental aberration could easily result in mission failure. It would not be too difficult to imagine a hypothetical martian NASA, undertaking to search for life on Earth, that had based its experiments on the average terrestrial water and thermal profiles but having the misfortune to land adjacent to a hydrogen sulfide-rich thermal hot spring. It is highly unlikely that life would be detected in these springs even though the environment literally teemed with life. A similar situation exists in the case of something as mundane as soil. It has been known for over 80 years that the vast bulk of the microorganisms present in soil fail to grow in the commonly used bacteriological media. Attempts to demonstrate their presence, using various physiological tests, turn out negative. Their presence was recognized by careful morphological studies followed by a wide variety of experiments which induced them to demonstrate their presence by subtle alterations of the growth environment.

If a returned martian sample contained a carbon/water based alien life-form, a whole set of experiments could lead to a reexamination of certain fundamental principles which biologists have accepted as necessary to describe life as we know it. These principles, while assumed to be universal, are limited by the restricted sample studied, an earth-based

life. It is not inconceivable that other principles have been employed, given a different planetary environment. The ability to examine extraterrestrial samples for life would provide the biologist with the same sort of test that has given the physicist and chemist the ability to make universal generalizations from terrestrial observations.

The availability of a martian biota thus would offer an opportunity to examine many of the terrestrial dogmas of biology. For example, the process of insuring that the cell produces an accurate copy of itself is generally considered an essential and necessary condition of life. Terrestrial life has accomplished the process by the use of DNA, a polymer consisting of 4 heterocyclic bases arranged in a 3 letter code. An examination of a martian biota's genetic material would be a critical test of the hypothesis that DNA serves as the repository of genetic material. In addition, it is not clear why of the many purine and pyrimidine bases found in nature, only 4 are found in genetic material. While it is possible that in some cases certain chemical and physical properties preclude the use of certain of these bases, this is not true for all cases. One could argue that many kinds of DNA bases were incorporated into primordial DNA and that through certain terrestrial selective pressures, only currently found ones have survived. Or it may be there exist first principles, which we fail to understand, that do not permit the use of these other bases.

It might be pointed out that prebiotic chemical simulation experiments yield numerous nucleic acid bases and amino acid monomers varying in physical structure and chemical composition. Yet only a few specific monomers are found in biological materials, suggesting the existence of some unknown selective factor or factors.

A similar situation exists in the case of the other major polymer found in the cell, proteins, whose function is primarily catalytic. Terrestrial proteins are composed of 20 amino acids, always in one optically active form, the l-configuration. It is not obvious why only 20 of the many amino acids found in nature can serve as protein building blocks, and why no proteins are known to contain a complete set of d-amino acids. As in the case of DNA, selective pressures may have been involved, eliminating all but the l-amino acid containing proteins. On the other hand, for

reasons unknown, proteins with amino acids in the d-configuration just cannot happen. Many more examples could be expanded upon: the universality of the mechanism of protein synthesis; the nature of cellular control; the nature of the limiting structure that encases the cell; the presence of unique energy yielding mechanisms. Studies of these areas, using terrestrial organisms, have led to a body of principles assumed not only to be characteristic but also **necessary** for life. The ability to examine extra-terrestrial forms of life would indeed be a critical test of universality.

A major stumbling block for any MSSR mission concerns the potential hazards to terrestrial biology associated with returning a martian surface sample. These possible hazards have been cogently reviewed by Martin Alexander (Quarantine for samples from Mars, in <u>Theory and Experiments in Exobiology</u>, II:123-146 (1972), ed. A. Schwartz). In theory, a quarantine procedure could be employed to isolate the martian surface sample until it is judged innocuous. The dilemmas associated with such a quarantine have been reviewed and it was suggested no practical quarantine procedure could be devised that could guarantee not a single terrestrial species would be infected by martian biota (see <u>Quarantine of Returned Mars Sample</u>, Report by L.I. Hochstein <u>et al</u>. to R.S. Young). One possible way out of this dilemma would be to sterilize the martian sample by chemical of physical agents which destroy critical cell function and/or structure.

Given the desirability of a MSSR mission, it is clear that only an unaltered sample would supply the maximum scientific information. If it contained viable organisms, the ability to culture them, so as to amplify their numbers, would permit a detailed study of their chemistry, function, structure, and environmental interaction. While in principle it would be possible to sterilize the sample and thereby prevent the proliferation of any indigenous organisms,this would necessarily result in an alteration of the very properties which give that material its uniqness - that is, the properties we associate with life and the universality of those properties. Furthermore, such treatment might so alter the structure and chemistry of the sample as to make a reconstruction difficult.

One need only imagine someone who never saw an egg attempting to reconstruct its structure, function, and chemistry

by viewing an omelet. While some chemical information could be obtained, most structural and biological information would be lost.

Considerations such as these suggested that a series of experiments be carried out to determine what manner of organic geo-chemical and morphological changes take place when terrestrial soil is subjected to various sterilization conditions and how serious is the degradation. As a first step in assessing the severity of scientific information loss arising from sterilizing treatments, a series of experiments were designed to address the following specific questions:

1. What is the minimum time and temperature required to sterilize a model soil?

2. What effect does dry heat sterilization have on the racemization and amino acid content of a model soil, rock and meteorite?

3. What effect does sterilization have on the morphology of the biological material?

4. Is it possible to chemically sterilize a soil without affecting the chemical and morphological parameters?

5. What are the synergestic effects of heat and radiation?

The results contained within this study constitute a preliminary answer to these questions. All of the parameters which might affect the observed results have not been investigated in detail, nor systematically, due to time considerations. If a decision is made to pursue the MSSR Mission, it is clear that it will require extensive studies of the kind presented and recommended in this report, particularly with respect to various soil types with different compositions and microbial loads.

SUMMARY

HEAT STERILIZATION

No bacterial survivors were detected when Hesperia soil was heated at 200°C for 24 hours. Hesperia soil could not be sterilized after heating at 125°C for 120 hours, or 150°C for 24 hours. The evidence for sterilization following heating at 150°C for 72 or 120 hours, or at 275°C for 24 hours was equivocal. Although bacterial growth was observed, the data could be taken to suggest that these putative survivors were contaminants.

It may be possible to sterilize soil at 150°C by heating for more than 24 hours, of at higher temperatures for shorter periods of time.

Soil can be rendered sterile be non-sterilizing dry heat exposure if the heat cycle is followed by an exposure to a non-sterilizing dose of gamma radiation.

CHEMICAL STERILIZATION WITH GLUTARALDEHYDE

2.5% glutaraldehyde did not sterilize Hesperia soil as evidenced by the profuse growth of fungi.

ORGANIC GEOCHEMISTRY OF HEAT STERILIZED SOIL, METEORITE, AND ROCK

No significant reduction of the total amino acid-nitrogen in soil was observed at 125°C or 150°C even after 120 hours of heating. Significant losses in total amino nitrogen was observed at 200°C (40%) and 275°C (80-90%) after 24 hours of heating. Analysis of the individual amino acids indicated that selective degradation had occurred. The most labile acids were the sulfur- and hydroxy-containing amino acids.

Of the 8 amino acids studied, only alanine, glutamic acid, and aspartic acid were extensively racemized when soil was heated at 150°C for 120 hours. Racemization of the amino acids was almost complete in the sample heated at 275°C for 24 hours.

The indigenous amino acids in the Murchison meteorite were examined following heating at 150, 200, and 275°C. In all cases, heating slightly altered the amino acid content, but only at 275°C were the alterations so great as to leave little useful chemical information. Some racemization was observed, but it was not extensive. These results are in good agreement with the previous studies made with soil, but are not in accord with the results observed with basalt (_vide infra_).

In a third experiment, a known mixture of L-amino acids was added to basalt, which in turn was subjected to various conditions of heating. Following heating at 150°C for 72 hours, or 200°C for 24 hours, the amino acid content dramatically decreased by about an order of magnitude. Heating at 275°C for 24 hours proved even more destructive. On the other hand, the extent of racemization, especially for temperatures at 200 and 275°C, was not as extensive as in soil.

These data suggest that the state of the amino acid (whether free or in some bound form) has a great influence on stability and ease of racemization.

ORGANIC GEOCHEMISTRY OF CHEMICALLY STERILIZED SOIL

Treatment of soil with osmium tetroxide or a mixture of glutaraldehyde, formaldehyde, and dinitrodifluorobenzene, resulted in a significant reduction of the amino acid content, but had little effect on racemization. Treatment with 6% glutaraldehyde resulted in little change in the amino acid content or the ease of racemization.

MORPHOLOGICAL EXAMINATION OF STERILIZED SOIL

Electron microscopy of soil heated at 150°C for 24 hours revealed the presence of flattened rods and spheres. These results suggest that heat had produced extensive morphological deformation.

In spite of the relatively large viable population present in the soil, it was difficult to observe microorganisms. This necessitated experiments in which soils were first treated with nutrients, so as to increase the microbial population, and subsequently subjected to 24 hours of heating at 200°C. While it proved easier to locate cells, they also underwent extensive morphological changes.

Chemical sterilization using a fixative consisting of glutaraldehyde, formaldehyde, and dinitrodifluorobenzene appeared effective.

RECOMMENDATIONS

STERILIZATION

1. That heat sterilization be studied with a wider variety of soils, media, and growth conditions.

2. That the effect of soil volume, mass, and relative humidity on the efficacy of sterilization be studied.

3. That the scoring techniques used for evaluating bacterial growth and survival be examined in more detail in order to detect extremely small numbers of survivors.

4. That the number of control and experimental samples required to achieve statistically significant information be determined.

5. That further studies of the synergistic effects of radiation and heat be carried out to determine the optimal conditions leading to sterilization and that these studies be carried out in conjunction with investigations on the combined effects of heat and radiation on the morphological and chemical properties of soil.

6. That subsequent investigations be conducted in an environment subjected to a minimal impact from exterior contamination. For example, the Ames Research Center Lunar Analysis Laboratory. This facility has a demonstrated record of contamination-free microbiological and chemical studies.

CHEMICAL STUDIES

1. That the stability of other organic substances of biological interest be investigated with respect to what effect heat and chemical sterilization may have on the information inherent in these molecules. Candidate classes of compounds are hydrocarbons, fatty acids, sugars, porphyrins, and nucleic acid bases.

2. That model studies be conducted to investigate what effect such factors as time, temperature of exposure, water content, and mineral matrix may have on the stability of compounds of biological and chemical interest employing a variety of soils, meteorites, and rocks.

3. That studies be initiated to devise more accurate models for martian surface materials. These models would be employed in further simulation studies.

4. That a number of ultrasensitive methods be developed for the preliminary evaluation of the organic chemical content of martian samples and that the effect of various sterilization procedures on the parameters these methods measure be evaluated.

MORPHOLOGICAL STUDIES

1. To develop more effective procedures for concentrating biological materials.

2. To ascertain what effect heating soils in the presence of fixatives may have on sterilization and morphology.

3. To develop techniques for analyzing soils for residual chemical sterilizing agents.

4. To further study the use of Electron Dispersive X-Ray Analysis as a probe for identifying the presence of microorganisms.

STUDIES ON THE CHEMICAL AND HEAT STERILIZATION OF SOIL

A Preliminary Study on the Relationship between Time and Temperature with Respect to the Sterilization of Soil

In response to a request from Dr. Richard S. Young, NASA Headquarters, certain members of the Biological Adaptation Branch (Planetary Biology Division, Ames Research Center) undertook to investigate the minimum time and temperature that would sterilize soil. These studies were carried out in conjunction with Dr. Delbert Philpott (Neurosciences Branch) and Dr. Keith Kvenvolden (Chemical Evolution Branch) who analyzed variously treated soils in order to determine what effect dry-heat sterilization had on the morphologic and chemical information contained within the soil.

Since there were definite time constraints for the completion of this project a number of necessary compromises in experimental design were made. To better define the limits of the data the following assumptions were made: (1) only spore-forming rods would survive sterilization; (2) the media were adequate for the growth of any survivors; (3) the number of replicate samples, while not statistically provident, was adequate for a preliminary study of this sort; (4) the method of preparing the soil was a fair approximation of dry-heat sterilization, although no attempt was made to control the moisture content; (5) the efficacy of sterilization was independent, to a first approximation, of sample size.

Soil was obtained from the Ames Research Center Soil Collection. It had previously been designated "Hesperia" indicating the region in the Southern Central Valley of California from where it was obtained. It is a neutral soil containing 2500 ppm organic material and 2% water (w/w). When first collected it contained a total of 7×10^6 aerobic bacteria of which 2×10^6 were aerobic spore forming rods (Table 1). Prior to use, the water content was redetermined by drying to constant weight at 108°C and found to be approximately 1.1% (w/w). The bacterial content of the soil capable of growing in the media used in these experiments was determined by standard plate counting techniques. The results of these experiments (Table 1) suggested that the values obtained when the soil was first prepared had changed slightly during storage. Anaerobic determinations were also made. The total number of anaerobes was 8.5×10^4/gm of soil of which 67% were spore formers (Table 1).

Table 2 describes the time-temperature matrix chosen for these studies. In order to test a particular time and temperature the following procedure was employed. Glass ampules of 10-ml capacity were rinsed with distilled water, dried, and heated for 45 hours at 275°C. Approximately 100 mg soil was added to each of 36 ampules and the ampules were evacuated to 10-20 mm Hg, using a water pump. After reaching the desired pressure they were flame sealed. For controls, 16 empty vials were evacuated,

flame-sealed, and then heated for 92 hours at 275°C. They were subsequently treated in the same manner as were the vials containing soil.

The vials containing soil and the empty control vials were set into racks, covered with aluminum foil, and placed in a circulating hot air oven preheated to the particular temperature to be tested.

The oven (monitored with a Yellow Springs Thermistor) was observed to maintain the desired temperature except when the doors of the oven were opened (not more than one per 24 hours). The subsequent loss of temperature within the vials was regained within 30 minutes, an inconsequential time, since the shortest period of heating was 24 hours. After heating, the vials were removed from the oven and placed in a sterile glove box that was continually flushed with nitrogen. Once cooled, they were opened aseptically and 5 ml of an appropriate medium was added. Standard soil extract medium (SE) was added to one set of 9 vials; to another set of 9, soil extract supplemented with 1% Difco tryptic soy broth (SES) was added. These 2 media were used to grow aerobic spore formers. To another set of 9 vials 5 ml of SE were added, while 5 ml of SES supplemented with 0.1% thioglycolate and 0.02% methylene blue was added to the remaining 9 (TSES). Following addition of the medium the latter 18 vials were sealed with VasPar in order to prevent the subsequent entrance of oxygen. These vials were used to test for the survival of anaerobic spore formers. All 36 vials were cotton plugged and incubated standing for 13 days at 24°C. After this period of time they were examined for turbidity as an indication of microbial growth. At this time the vials were shaken, incubated for an additional period of 24 hours and 1 ml of growth medium was removed from the vials and plated on medium identical to the medium in the vials except that 2% agar was added as a solidifying agent. The plates were subsequently incubated at 24°C (the aerobic samples in polyethylene bags to prevent evaporation, the anaerobic plates in Brewer jars) for 2 weeks at which time they were scored for the presence of microbial growth. Immediately after plating, the contents of those vials which were turbid were examined microscopically to insure that the turbidity was due to the presence of bacteria. Thus the test for survival was three-fold: the vials were examined for turbidity as evidence of microbial growth; growth on solid medium (to detect survivors which were present at too small a concentration to give rise to visible turbidity); and microscopic examination of the turbid vials to ascertain that turbidity was due to microbial forms.

The 16 controls, divided into 4 sets of 4, one set of 4 incubated aerobically in each of the 2 "aerobic media" and one set of 4 incubated anaerobically in each of 2 "anaerobic media" were treated in an identical manner.

Table 3 is a flow sheet which describes the manner an experiment was carried out for a particular time and temperature.

RESULTS

The data presented in Tables 4 and 5 represent a summary of these experiments. The results are given as the number of positive vials or plates that were observed out of 9 tested (i.e., exhibiting turbidity

or growth, either as a lawn or discrete colonies). Since there were more positive vials and plates using SES and TSES media, only those results will be discussed. In no case was growth ever observed in SE but not SES or TSES medium.

The data for the controls are not presented because only 2 of the 128 controls showed growth, and they were identified as molds. One appeared on a plate from a vial that was heated at 125°C for 120 hours; the other appeared on a plate from a vial heated at 275°C for 24 hours.

As shown in Table 4, heating Hesperia soil at 125°C for 24, 72, and 120 hours was insufficient to sterilize it. After heating for 24 hours aerobic incubation resulted in 8 of the vials being turbid, of which 6 gave rise to visible growth on solid medium. When Hesperia soil was heated for 72 and 120 hours at 125°C, all of the vials and plates were scored as containing viable organisms. At the present time, there is no obvious explanation why the 2 turbid vials failed to produce visible growth on the solid medium as in both instances the presence of microorganisms was verified microscopically in the vials.

As shown in Table 5, 7 vials that were heated at 125°C for 24 hours produced turbidity when incubated anaerobically. However, only 1 of these vials produced visible growth on plates.

After heating for 24 hours at 150°C, 1 vial was turbid (Table 4). Microscopic examination of the contents of this vial revealed the possible presence of organisms that were, however, difficult to distinguish from soil particles. Three non-turbid vials that were heated at 150°C for 24 hours produced growth on SES plates. However, no growth was observed in the plate prepared from the turbid vial. One of the plates contained yellow colonies which on microscopic examination proved to be tetrads, possibly a member of the genus Sarcina. A second plate contained a lawn consisting of unpigmented growth. The third plate had about 50 colonies consisting of 3 colonial types. One was yellow and probably similar to the yellow Sarcina-type observed previously. The second was orange; microscopic examination revealed the presence of short, fat rods. The third type of colony was white and consisted of small rods.

After heating at 150°C for 72 hours, none of the vials was turbid. However, 2 SES plates were observed to contain 1 colony each. One of these had a yellow surface on top of which was "sprinkled" a whitish material. The remaining portion of the colony, which was slightly translucent and pearl white in color, appeared to extend into the surface of the agar. Microscopically, the colony contained filamentous forms. The presence of the whitish material on the surface of the colony, its filamentous character, and the distinct musty odor produced by the colony suggested that it might be Streptomyces. The colony on the other plate was white and had an irregular shape. Microscopic examination revealed that it contained very small pleomorphic rods.

Since Streptomyces are not known to produce heat-resistant forms, either this organism is a contaminant or, intriguingly and conservative, representative of soil bacteria that have not been previously recognized to produce heat resistant stages. The Sarcina-type probably is a member of the motile sarcinoids which are known to produce heat resistant spores.

None of the vials containing soil that was heated for 120 hours at 150°C was turbid after 13 days of incubation (Tables 4 and 5). However, 1 plate was found to contain 2 colonies. One was bacterial and consisted of pleomorphic, motile rods; the other was a mold as evidenced by the presence of septate hypae. None of the anaerobic plates were found to contain bacterial colonies (Table 5).

As shown in Tables 4 and 5, none of the vials containing soil heated at 200 or 275°C for 24 hours was turbid. Examination of the plates prepared from these vials revealed that single plate, derived from a vial that was heated at 275°C for 24 hours, contained a white colony that had spread over a large portion of the plate. The significance of this colony is difficult to assess at the present time since it is not obvious why an organism that can survive heating at 275°C for 24 hours did not survive heating for 24 hours at 200°C.

These results indicate that it is not possible to sterilize Hesperia soil by heating it at 125°C for as long as 120 hours or 150°C for 24 hours. At 125°C the heat-resistant population consisted of aerobic and anaerobic types. At 150°C only aerobic organisms were observed, one of which appears to be a member of what formerly was designated as Sporosarcina. Another organism was tentatively identified as Streptomyces—none of the other isolates has been identified as yet. No evidence for the presence of organisms surviving heating after 200°C for 24 hours was obtained. These data can be taken to suggest that Hesperia soil may not be heat-sterilized at 150°C for 24 hours.

CAVEATS

It is necessary to point out that these results cannot be taken to state unequivocally that a temperature and time where no evidence of growth was obtained (i.e., at 200°C) sterilizes soil for the following reasons.

(1) The limited kinds of media employed in this study (3) may have failed to support the growth of heat-resistant bacteria which survived these temperatures and times.

(2) More anaerobes may have survived than would be indicated by the results since the presence of thioglycolate and methylene blue in the TSES medium may have been toxic for anaerobic heat-resistant survivors.

(3) Only 2 degrees of oxygen tension were tested, 1 which would select for aerobes and the other for anaerobes. The possibility exists that heat-resistant survivors may require some intermediate oxygen tension, or even oxygen tensions more or less than the extremes employed.

(4) The scoring technique for recognizing resistance to heat sterilization depended in part on being able to observe the presence of turbidity. If insufficient numbers of survivors were present, coupled with their failure to grow on solid medium, the presence of heat-resistant survivors would have been missed. That this situation is possible is suggested by the results obtained with the soils heated at 150°C for 24 hours. The one tube that was turbid failed to give rise to visible growth when a portion of its contents were plated on solid medium. And 3 vials which were not turbid did produce growth on solid medium.

(5) There may have been heat-resistant bacteria present which grew too slowly to produce a detectable turbidity during the limited incubation period and whose numbers were so small that the subsequent sampling for growth on solid medium may have failed to carry any (or enough) over to produce detectable growth on solid medium.

(6) The number of samples employed in this experiment was relatively small. In particular, the number of controls should have at least equalled the number of "experimental" vials. This is of particular concern since in the case of the soils heated at 150°C for 72 hours only a single colony was observed on each of 2 SES plates. Since at least one of these colonies appeared to be a bacterium not usually thought of as heat resistant, one is faced with the possibility that: 1) it was not unusually heat-resistant but happened to come from the tail-end of a normal distribution curve of temperature versus survivors; 2) it may represent a contaminant; 3) it may indeed be an unusually heat-resistant species.

(7) The relative humidity was not controlled during heat sterilization so that it was not the same in all the vials regardless of the temperature of sterilization. Since relative humidity markedly affects resistance to heat sterilization this parameter may have had a profound effect on the results described.

(8) The results obtained in this study apply only to the particular soil that was employed. It is not clear what effect such factors as the organic content (qualitative and quantitative), microbial load, the physical nature of the soil (i.e., clay, loam, sandy), water content, pH, inorganic content, and ion composition could have on the resistance of organisms to heat sterilization.

CHEMICAL STERILIZATION OF SOIL

An experiment was carried out to determine whether it was possible to sterilize Hesperia soil with glutaraldehyde. The protocol employed was essentially that described by Dr. Earl Casada, Pennsylvania State University.

Two 5-gram quantities of Hesperia soil were added to each of 2 sterile 300-ml polycarbonate centrifuge bottles. Subsequently 250 ml of sterile 25 mM potassium phosphate buffer (pH 7.5), 2.5% with respect to glutaraldehyde, was added. The soil-glutaraldehyde mixture was manually shaken for about 5 minutes and incubated at room temperature for 17 hours. Residual glutaraldehyde was removed by serial centrifugation at 10,000 × g for 30 minutes. The supernatant was decanted and the

sedimented soil (and microorganisms) resuspended in 250 ml of sterile
25 mM potassium phosphate buffer, pH 7.5 buffer. This procedure was
repeated for a total of 4 times. After the last wash 89 mg of moist
soil were spooned into each of 10 tubes of sterile tryptic soy broth
made up in soil extract medium. The tubes were incubated at 27°C for
1 month. Sterility controls were prepared by opening sterile tubes of
1% tryptic soy broth-soil extract and inoculating them with an empty
sterile dispensingspoon similar to the one used previously to dispense
the glutaraldehyde-treated soil. In addition, 2 other controls were
run. One contained untreated soil in order to determine whether the
medium employed would support the growth of organisms. The other
control consisted of untreated soil and a spoonful of the last wash
from the glutaraldehyde-treated soil. This control was used to
determine whether there was sufficient glutaraldehyde present in the
final wash to inhibit the growth of microorganisms.

After 3 days of incubation, all the vials containing glutaraldehyde-
treated soil contained fungal growth. Microscopic examination did not
reveal the presence of any bacteria. None of the sterility controls
were contaminated. The control used to test the efficacy of the medium
to support microbial growth and the control used to determine whether
there was an inhibitory carry-over of glutaraldehyde were grossly
contaminated with bacterial growth. After 1 month of incubation, none
of the sterility controls were contaminated.

These results indicate that it is not possible to sterilize Hes-
peria soil using 2.5% glutaraldehyde, pH 7.5 at 22°C.

TABLE 1

PROPERTIES OF HESPERIA SOIL

Air-Dried

Sieved through a 2 mm screen

Organic Content   2500 ppm

Water Content   . 1.1% (w/w)

Bacterial Count (colony forming units/Gm soil)

   1. Original

      A. Total 7×10^6

      B. Aerobic Spore Formers 2×10^6

   2. As determined 2/14/74

	MEDIUM	
	SE	SES
A. Total Aerobic	1.6×10^6	5.8×10^6
B. Aerobic Spore Formers	2.2×10^4	3.2×10^5
C. Total Anaerobic	1.0×10^3	8.5×10^4
D. Total Anaerobic Spore Formers	1.0×10^2	5.7×10^4

Table 2

MATRIX OF TIMES AND TEMPERATURES TESTED

TIMES (HRS)	TEMPERATURES (C)			
	125	150	200	275
24	+	+	+	+
72	+	+	NR*	NR
120	+	+	NR	NR

*Not run.

TABLE 3

FLOW SHEET OF EXPERIMENTAL PROCEDURE

SOIL SAMPLE

INCUBATE AT 25 C IN:

```
                    AEROBIC              ANAEROBIC
                     MEDIA                 MEDIA

              SE          SES         SE          TSES



        OBSERVE FOR TURBIDITY


           (+)                      (-)


EXAMINE MICROSCOPICALLY             PLATE


  (+)              (-)        (+)              (-)
```
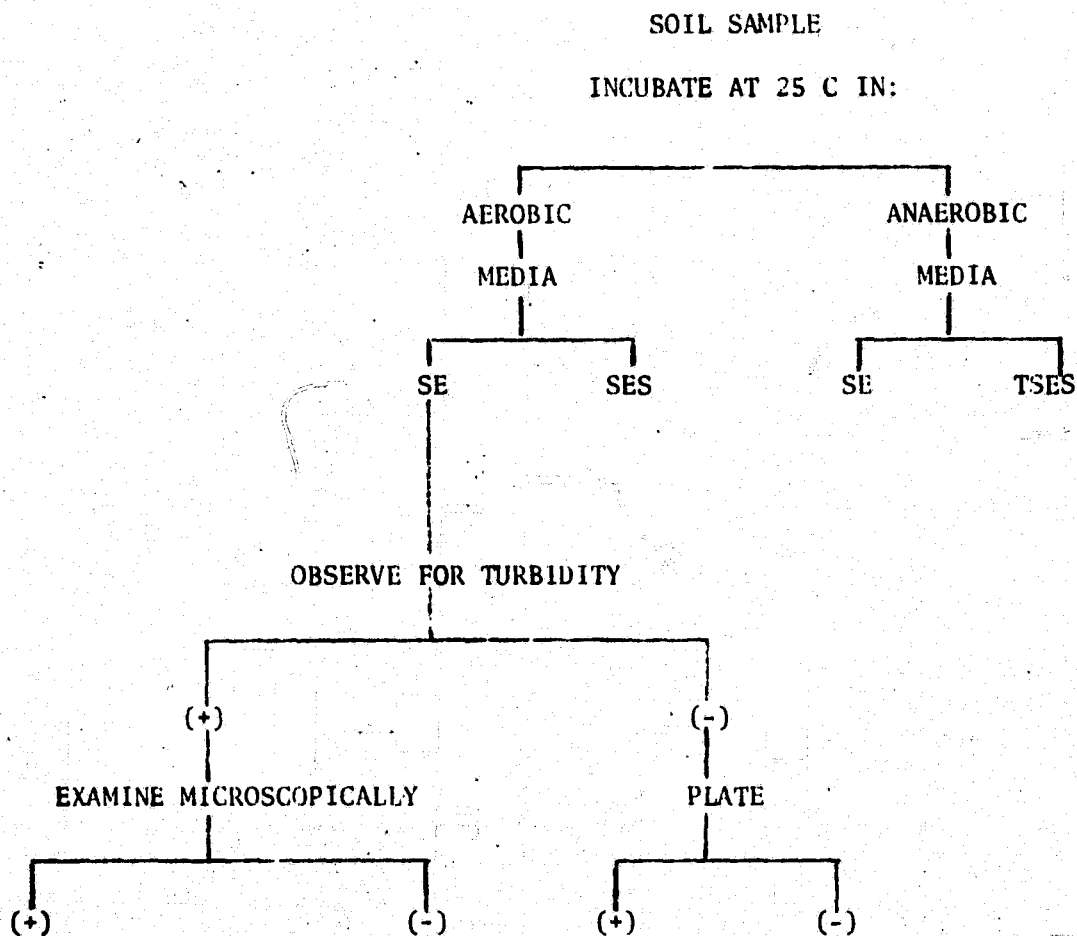
NOTE: The dichotomy presented describes only the series of aerobic

incubations in SE medium. It is similar in the case of the

other 3 branches.

Growth was considered to have taken place whenever a branch

point is positive.

Table 4

TURBIDITY IN THE VIALS INCUBATED AEROBICALLY

TEMP TIME	SE MEDIUM				SES MEDIUM			
	125	150	200	275	125	150	200	275
24	5	0	0	0	8	1	0	0
72	0	0	NR	NR	9	0	NR	NR
120	0	0	NR	NR	9	0	NR	NR

GROWTH ON PLATES INCUBATED AEROBICALLY

TEMP TIME	SE MEDIUM				SES MEDIUM			
	125	150	200	275	125	150	200	275
24	2	0	0	0	6	3	0	1
72	0	0	NR	NR	9	2	NR	NR
120	0	0	NR	NR	9	0	NR	NR

Table 5

TURBIDITY IN THE VIALS INCUBATED ANAEROBICALLY

TIME \ TEMP	SE MEDIUM				TSES MEDIUM*			
	125	150	200	275	125	150	200	275
24	2	0	0	0	7	0	0	0
72	0	0	NR	NR	0	0	NR	NR
120	0	0	NR	NR	0	0	NR	NR

*SES MEDIUM + 0.1% thioglycolate + 0.02% methylene blue

GROWTH ON PLATES INCUBATED ANAEROBICALLY

TIME \ TEMP	SE MEDIUM				TSES MEDIUM*			
	125	150	200	275	125	150	200	275
24	0	0			1	0	0	0
72	0	0	NR	NR	0	0	NR	NR
120	0	0	NR	NR	0	0	NR	NR

*SES MEDIUM + 0.1% thioglycolate + 0.02% methylene blue

Effects of Heat and Chemical Sterilization on the Amino
Acids in Simulated Returned Martian Samples

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Abstract

It may be necessary and/or required to sterilize the first surface samples of Mars returned to Earth. The effects of sterilization procedures on simulated martian samples, therefore, need to be evaluated in order to determine to what extent organic geochemical information may be altered in returned martian samples. In this study heat sterilization procedures have been shown to alter amino acid populations in three simulated returned martian samples -- basalt plus a mixture of eight standard L-amino acids and glycine, carbonaceous meteorite, and soil. The degree of alteration varies with each sample. The behavior of amino acids in the meteorite and soil is similar under heat sterilization conditions at 125° and 150°C for 24, 72 and 120 hours. At these conditions there is little change in amino acid populations and the extent of racemization is minimal except for those amino acids which are particularly labile to racemization. At 200°C for 24 hours amino acids, particularly in the soil, show measurable alterations, and at 275°C for 24 hours extensive destruction of amino acids renders both the meteorite and soil samples almost useless. Chemical sterilization applied to the soil reduced the amino acid content, but caused little racemization. In contrast to the meteorite and soil the amounts of amino acids in the basalt are significantly reduced even when the samples are treated at 150°C for 72 hours, but the degree of racemization is not great.

Introduction

The object of this work is to determine to what extent organic geochemical information in returned martian samples will be altered by sterilization procedures. In this preliminary report we focus on changes in the concentrations of amino acids and their enantiomers (optical isomers) that take place when simulated martian samples are sterilized under various conditions. Although ultimately a number of different classes of biologically significant organic compounds must be examined in the context of this work, we have selected, at this time, amino acids because of their importance in biology, chemical evolution and organic geochemistry and because our previous experience with these kinds of molecules has been extensive. The work reported here began in February 1974, and to date represents an expenditure of eight man-months of effort.

Simulated Martian Samples

The following sample types were selected for these simulation studies:

(a) W-1 _ U.S.G.S. diabase basalt standard _ pulverized samples were used as received from the U.S. Geological Survey after they were homogenized and quartered on glassine paper.

(b) Limonite _ Iron oxide _ Powdered sample was a variety of Yellow Ochre from Cartersville, Georgia, and was supplied by Ward's Natural Science Establishment. --

(c) Montmorillonite _ Clay _ Sample was from Otay, California and was also supplied by Ward's Natural Science Establishment. Sample was pulverized and ground using a steel lined shatter-box and alumina mortar and pestle. Sample was seived to less than 62.5 μm. and homogenized.

(d) Murchison meteorite _ C2 carbonaceous chondrite _ Sample furnished by Dr. Ed Olson, Chicago Museum of Natural History. Sample prepared with the same procedure used for montmoril-lonite.

(e) Hesperia _ Soil _ Sample was taken from an agricultural area near Bakersfield, California and was furnished by Dr. Ed Merek, Ames Research Center. It was air dried and passed through 2mm sieve The sample contained 1% by weight of water and 2500 ppm organic carbon.

Sample Preparation

For our particular purposes a standard mixture of eight L-amino acids plus glycine (total concentration of 1.19 mg/ml) (Table 1) was added to (a) W-1 basalt, (b) limonite, and (c) montmorillonite. We assumed that any indigenous population of amino acids in these samples would be near or below the limits of detection of our analytical techniques. The standard amino acids were dissolved in 0.1 N HCl, and one ml of this solution was added to each of four approximately one gram portions of each sample. The samples were dried in a vacuum oven at about 38°C. These portions were each gently repulverized and homogenized with an alumina mortar and pestle.

Samples of Murchison meteorite and Hesperia soil were not treated with the standard amino acid solution.

Sterilization Procedures Applied to W-1 Basalt, Limonite, Montmorillonite and Murchison Meteorite

To provide a tie to concurrent inorganic analyses of these samples, E.A. King, University of Houston, prepared the samples of W-1 basalt, limonite, montmorillonite and meteorite and applied the following sterilization conditions: "Splits of approximately one gram each of all samples were heated in a helium atmosphere to 150° for 72 hours, 200° for 24 hours and 275°C for 24 hours. In all runs the samples were placed in the furnace and brought up to temperature, held at the desired temperature and cooled to room temperature under a constantly flowing helium atmosphere. After heating the cool samples were removed from the furnace and helium atmosphere and were sealed in glass vials with screw caps. Samples containing the organic spikes had clean aluminum liners placed under the screw caps, thereby hopefully avoiding contamination from the plastic top and cardboard insert." Unheated portions of these samples were used as controls. Table 2 summarizes the conditions of temperature and time applied to these samples.

Sterilization Procedures Applied to Hesperia Soil

Both heat and chemical sterilization procedures were applied to samples of Hesperia soil. Organic geochemical studies of these samples provided a tie to concurrent studies in microbiology and biological morphology. Heat sterilization of samples for organic geochemical analyses was accomplished at the same time and under the same conditions as used in preparation of samples for microbiological studies. The protocol for this sample was established by L.I. Hochstein. Table 3 shows the time-temperature matrix used in the procedure for testing sterility. Approximately one gram portions of soil were placed in glass tubes and evacuated to 10-20mm Hg and sealed. The tubes were placed in baskets in an oven the temperature of which was monitored and recorded during the heating process.

Chemical sterilization methods were also tested. One sample was treated with 2.5 percent glutaraldehyde by L.I. Hochstein; other samples of soil were treated by D. Philpott with a 6 percent solution of glutaraldehyde, a solution of osmium tetroxide and a solution called Triple-fix (glutaraldehyde, formaldehyde and dinitrodifluorobenzene).

Analyses

W-1 Basalt and Murchison Meteorite

Although four different sets of samples (W-1 basalt,
limonite, montmorillonite, and Murchison meteorite) were to be
evaluated, only two sets of samples have been analyzed to date.
The samples were hydrolyzed directly with 6N HCl. The hydrolysate
was recovered and desalted by ion exchange methods (Dowex 50). The
amino acid containing fraction was evaporated, and the resulting
amino acids were converted to volatile diastereomeric derivatives.
These derivatives were analyzed by gas chromatography (GC) in order
to obtain an approximate measure of amino acid concentration and
to determine the ratio of amino acid enantiomers. Figure 1 shows the
scheme of analyses for these samples.

Hesperia Soil

Samples of Hesperia soil were subjected to a scheme of analysis which provided a measure of (1) the concentration of individual amino acids in the soil, (2) the total amino acid nitrogen, and (3) the ratio of amino acid enantiomers. Samples were hydrolyzed with 6N HCl. A portion of the resulting hydrolysate (0.1g equivalent) was examined for total amino acid nitrogen by a modified ninhydrin method developed by Cheng and Stevenson (Geochim. Cosmochim. Acta. 34:77(1970)). The remainder of the hydrolysate was desalted by ion exchange methods (Dowex-1 F$^-$) and the resulting amino acid fraction was divided into two parts. One part (0.4g equivalent) was analyzed by conventional automated ion exchange chromatography (CIE), and the other portion (0.5g equivalent) was desalted by ion exchange (Dowex 50 H$^+$) and the resulting amino acids were converted to volatile diastereomeric derivatives which were analyzed by gas chromatography (GC). The scheme of analysis for Hesperia soil is shown in Figure 2.

Results

W-1 Basalt

Preliminary results on W-1 basalt show that the concentrations of the amino acids in the heat treated samples diminish dramatically. Table 4 compares the amino acids in the control and three heat treated samples. There is little difference in the concentrations of amino acids in samples heated at 150 and 200°C. The amounts of amino acids are in all cases, except one, at least an order of magnitude less than in the control. The amino acids in the sample heated at 275°C are detectable but in most cases are two orders of magnitude less abundant than in the control. Figure 3 shows the relative amounts of amino acids in these samples.

Heat treatment increases the degree of racemization but not extensively. The percentages of D-amino acids for the optically active amino acids are shown in Table 5. The apparent percentages of D-amino acids in the control range from 1.3 to 2.5. The highest percentages of D-amino acid are found in the sample treated at 275°C where D-alanine is 16.1%, D-aspartic acid is 15.4% and D-glutamic acid is 16.6%. Determination of the percentages of D-amino acids in this sample is difficult, because so little of the original amino acids remain.

Murchison Meteorite

Results from the Murchison meteorite can best be discribed by the gas chromatographic patterns of the amino acid fractions. Figures 4 and 5 compare the amino acids in the control with the sample treated at 275°C. Not only has the concentration of identifiable compounds diminished, but the pattern has altered severely, and little interpretable information remains. New compounds having long gas chromatographic retention times are generated during the heating process. The generation of materials having long retention times appears even in samples treated at 150°C.

The percentages of D-amino acids can be calculated for only a few of the amino acids because of the general complexity of chromatograms. Table 6 lists the percentage of D-amino acids obtained Some racemization takes place but it has not proceeded to completion in the samples treated at 150 and 200°C. For the sample heated to 275°C, few amino acids could be identified; therefore the degree of racemization could not be ascertained.

Hesperia Soil - Total Amino Acid Nitrogen

Total amino acid nitrogen values for the control and heated samples of Hesperia soil are shown in Table 7. Very little or no change occurs in the amino acid nitrogen in samples treated at 150°C for 24 to 120 hours For samples heated at 200°C, however, a 40 percent reduction in amino acid nitrogen is observed. The sample treated at 275°C for 24 hours showed an 80 percent loss of amino acid nitrogen.

Soil samples which had been subjected to chemical sterilization procedures were also examined for their content of total amino acid nitrogen, and the results are shown in Table 8. The amino acid nitrogen determined for the sample treated with dilute glutaraldehyde solution significantly exceed the amino nitrogen of the control. This result suggests that the glutaraldehyde did not completely sterilize the sample, and apparently promoted the growth of organisms. With concentrated glutaraldehyde the sample showed no increase in amino nitrogen. Osmium tetroxide and Triple-fix apparently cause a partial loss of amino acids in the samples to which these solutions were applied.

Hesperia Soil - Conventional Automated Ion Exchange Chromatography (CIE)

Table 9 records the results obtained for individual amino acids as determined by CIE procedures. With the exception of hydroxy-and sulfur-containing amino acids, little variation in amino acid concentrations occurs in samples treated at temperatures below $200^{\circ}C$. At $200^{\circ}C$ some losses of amino acids can be observed, but at $275^{\circ}C$ about 90 percent of the original amino acids are lost. Figure 6 summarizes approximately the variations in the total amino acids as a function of sterilization treatment.

The sample treated with dilute glutaraldehyde contained more amino acids than the control. This result confirms the observation made with acid nitrogen determinations. Samples treated with osmium tetroxide and Triple-fix showed loss of amino acids; the greatest loss occurs with osmium tetroxide.

Hesperia Soil - Ratios of Amino Acid Enantiomers

Ratios of amino acid enantiomers are reported as the percentages of D-amino acid as a function of temperature-time and chemical treatments. Eight optically active amino acids were investigated, and the results are shown in Table 10. With the exception of aspartic acid, the degree of racemization changes only slightly for samples treated at 125 and 150°C for 24 to 120 hours. Under these temperature-time conditions the extent of racemization of aspartic acid changes from 8.6% D-aspartic acid in the control to 24.8% D-aspartic acid at 150°C-120 hours. The sample treated at 200°C for 24 hours showed significant, but incomplete racemization; at 275°C for 24 hours racemization of some of the residual amino acids is almost complete.

Figures 7 and 8 compare the gas chromatographic results on the amino acids in the control and in the sample treated at 275°C for 24 hours; the loss of amino acids and the racemization of the residual amino acids is clearly illustrated.

Discussion

The results obtained from this preliminary study do not permit broad
generalizations to be made with regard to the possible effects of sterilization on
returned martian samples. The results do, however, establish some guides and
set some limits on future work.

W-1 Basalt

This sample represents a possible matrix material which may be encountered
on the martian surface. Free amino acids in hydrochloric acid solution were
added to portions of this sample, and, therefore, these compounds likely
existed in the free hydrochloride form. It is not possible to say if there existed
any interaction between the amino acids and the minerals of the basalt. The
rapid decrease in concentration of the amino acids upon heating suggests that the
mineral matrix provided little protection from the degradation process, although
the extent of destruction of these amino acids alone without basalt was not
determined. The extensive destruction of the free amino acids in the treated
basalts contrasts with the results obtained from the Murchison meteorite and
from Hesperia soil. In these latter samples some losses were observed at
200°C and below, but not extensive losses. The contrasting observations
suggest that the state of the amino acids in the matrix, and possibly the matrix
itself, have great influence on the stability of amino acids under heat treatment.
The indigenous amino acids in Murchison meteorite and Hesperia soil must be
intimately associated with the mineral matrix.

Although heat treatment increased the extent of racemization of amino acids, the increase was not extensive, reaching a maximum of about 17 percent in the sample treated at 275° for 24 hours. There is little evidence for metal ion catalysis of the racemization reaction; had much water been present, the extent of racemization likely would have been much higher. Amino acids in Murchison meteorite and particularly in Hesperia soil at temperatures of 200 and 275°C were more extensively racemized than were the free amino acids in basalt. It appears that the amino acids bound in a mineral matrix such as meteorite and soil are more stable to heat treatment, but undergo racemization more quickly than do free amino acids at higher temperatures.

Murchison Meteorite

Results from the Murchison meteorite are difficult to interpret because of the complexity of the chromatograms. The samples examined were all contaminated with terrestrial amino acids, because no special precautions were taken during sample preparation to remove the outer surfaces which contained amino acids from individuals who have handled the sample. In contrast to uncontaminated samples of Murchison meteorite that contain racemic mixtures of amino acids, these samples all showed a dominance of L-amino acids. Apparently the amino acids observed in the control represent the indigenous amino acid population plus the contaminating L-amino acids from sample handling. There are advantages and disadvantages to working with contaminated samples of meteorite. With contaminated samples it is possible to follow the extent of racemization upon heat treatment; with uncontaminated samples amino acids are already present as racemic mixtures. Only changes in amino acid compositions can be determined. On the other hand contaminated samples contain two populations of amino acids each of which is likely to be bound in the mineral matrix in a different manner. Under heat treatment these populations of amino acids may behave differently thus increasing the difficulty of interpretation of results.

Heat treatment alters the composition of the amino acids in the Murchison meteorite but for samples heated at 150 and 200°C for 24 hours, the amino acid chromatographic patterns remained similar although racemization is extensive relative to the racemization of free amino acid in the basalt samples. Only at 275°C for 24 hours does the treatment result in a sample which appears to be *useless*

Hesperia Soil

Heat treatments of the soil did not alter the amino acid content of these samples until the condition of 200°C was applied for 24 hours. At this temperature- time condition there was a 40 percent reduction in amino acid nitrogen, and the total amino acid concentration determined by CIN methods reduced about 25 percent. That the figures for amino acid nitrogen and total amino acids are not more nearly equivalent is not considered significant. The important point is that both figures show a measurable loss of amino acids at 200°C.

At temperature-time conditions below 200°C and 24 hours racemization of amino acids did occur. For amino acids such as leucine, isoleucine, proline and phenylalanine the extent of racemization is low. Alanine, aspartic acid and glutamic acid however, undergo significant racemization. For example, in the sample treated at 150°C for 120 hours, the %D-aspartic acid equalled 24.8. At 200°C and 24 hours the amino acids in the soil were incompletely racemized; percentages of D-amino acids varied from 8.8 for proline to 33.3 for aspartic acid. The small degree of racemization of amino acids in samples heated at 125 and 150°C may be due to the low moisture content in the soils (about 1 percent). If the soil had been completely dry before heat treatment even less racemization might have occurred. On the other hand

if the soil had been wet, racemization likely would have been more pronounced.

Amino acids in the sample treated at 275°C for 24 hours were extensively destroyed and extensively racemized. If Hesperia soil is a proper analogue for a martian sample, it seems that heat sterilization at 275°C for 24 hours would definitely not be an acceptable procedure.

Chemical treatment of soils shows that concentrated glutaraldehyde, osmium tetroxide and Triple-fix all decrease the content of amino acids. Osmium tetroxide was particularly destructive. These chemical methods appear to destroy more amino acids than any of the heat treatments below 200°C. The chemical treatments caused little or no racemization of amino acids, however. Dilute glutaraldehyde solution caused an increase in the amino acid concentration from the soil; it appears that the solution produced a medium for the growth of organisms thus causing an increase in the amino acids recoverable from the soil.

Conclusions

Heat and chemical sterilization procedures have been shown to alter the amino acid populations in three simulated returned martian surface samples -- basalt, carbonaceous meteorite and soil. The degree of alteration varies with each sample.

1) Basalt - The basalt contained a suite of L-amino acids which was added to the sample before heat treatment at 150° for 72 hours and 200°, and 275°C for 24 hours each. At all temperature-time conditions the amino acid concentrations were reduced dramatically. At 200° and 275°C the amount of residual amino acids were respectively about one and two orders of magnitude less than in the control. Racemization was not extensive. The highest degree of racemization was measured at 16.6% D-glutamic acid in the sample treated at 275°C.

2) Murchison meteorite - This sample contained an indigenous population of amino acids plus an overprint of contaminating L-amino acids. Portions of the sample were treated at the same temperature-time conditions as was the basalt. Up to 200°C there appears to be little change in the concentration or distribution of amino acids; at 275°C the concentration of amino acids has diminished and little interpretable information remains. Amino acids in the meteorite racemized to a greater extent than those in the basalt; extent of racemization of amino acids in the meteorite treated at 275°C could not be ascertained.

3) Hesperia soil - This sample contained mainly an indigenous population of amino acids. Portions of the sample were treated at 125° for 24, 72 and 120 hours, at 150° for the same time period, at 200° and 275°C and for 24 hours.

No significant reduction of total amino acids was observed at temperatures of 125° and 150°C. At 200°C some measurable losses occur, but at 275°C about 85% of the amino acids are lost. The degree of racemization changes only slightly for samples heated at 125 and 150°C except for aspartic acid. The sample treated at 200°C showed significant but incomplete racemization of all amino acids; at 275°C racemization of some of the amino acids was almost complete. Soil samples chemically treated with concentrated glutaraldehyde, osmium tetroxide and Triple-fix showed a reduction of the amino acid content, but little racemization occurred.

The behavior of amino acids under heat sterilization conditions at 125°and 150°C in the meteorite and soil is similar. At these conditions there is little change in the amino acid populations and the extent of racemization is minimal except for those amino acids, such as aspartic acid, which are particularly labile to racemization. At 200°C amino acids, particularly in the soil, show measurable alteration, and at 275°C the extensive amino acids destruction renders the samples useless.

In contrast to the meteorite and soil the amounts of amino acids in basalt are signficantly reduced even at the temperature of 150°C. The basalt may provide a poor simulation of a returned martian sample in that amino acids are in the basalt as free hydrochlorides. Their rapid alteration may be due to a lack of an intimate relationship with the mineral matrix. Results obtained from the meteorite and soil are believed to provide a better estimate of the alterations in amino acids that might occur in a heat sterilized, returned martian surface samp

From these preliminary observations the following suggestions can be made:

(1) Sterilization of a returned martian sample should be avoided if at all possible. Although the amount of alteration is small at 125 and 150°C, some changes in chemical composition do take place.

(2) If sterilization is necessary, dry-heat sterilization at the lowest possible temperatures should be used. Chemical steri-. lization procedures studied here do not appear to be quite as attractive as heat sterilization.

(3) The preliminary data show that the upper limit for heat sterilization with minimum alteration is about 150°C for a period not to exceed about 120 hours. Interpolation, however, of the present data suggests that higher temperatures of about 175°C may possibly be used if the time for sterilization does not exceed 72 hours. Sterilization at these or similar temperature-time conditions should be evaluated.

(4) Useful information is still retained in samples heated to 200°C for 24 hours but it must be recognized that significant alteration occurs at these conditions.

TABLE 1

AMINO ACIDS ADDED TO SIMULATED MARTIAN SAMPLES

L-ALANINE	— 0.144 mg/ml
L-VALINE	— 0.093 "
L-ISOLEUCINE	— 0.118 "
L-LEUCINE	— 0.112 "
L-PROLINE	— 0.139 "
L-ASPARTIC ACID	— 0.156 "
L-PHENYLALANINE	— 0.172 "
L-GLUTAMIC ACID	— 0.128 "

AMINO ACIDS WERE SUPPLIED BY THE
AJINOMOTO COMPANY.

TABLE 2
HEAT STERILIZATION OF W-1 BASALT, LIMONITE, MONTMORILLONITE AND MURCHISON METEORITE

	CONTROL	275° 24 hr	200° 24 hr	150° 72 hr
W-1 (PLUS STANDARD AMINO ACIDS)	X	X	X	X
LIMONITE (PLUS STANDARD AMINO ACIDS)	X	X	X	X
MONTMORILLONITE (PLUS STANDARD AMINO ACIDS)	X	X	X	X
MURCHISON METEORITE	X	X	X	X

HESPERIA SOIL WAS ALSO TREATED WITH THIS TEMPERATURE-TIME PROTOCOL, BUT THESE SAMPLES WERE NOT CONSIDERED FOR THIS REPORT.

TABLE 3

TIME-TEMPERATURE MATRIX FOR HEAT STERILIZATION OF HESPERIA SOIL

TIME (hr)	TEMPERATURE °C			
	125°	150°	200°	275°
24	X	X	X	X
72	X	X		
120	X	X		

TABLE 4
AMINO ACIDS RECOVERED FROM W-1 BASALT
(PERCENT RECOVERED RELATIVE TO CONTROL)

| | TEMPERATURE °C / TIME (hr) | | |
	CONTROL	150°/72	200°/24	275°/24
VALINE	100	5.8	6.6	3.9
ALANINE	100	5.7	6.7	0.54
ISOLEUCINE	100	1.7	2.4	—
LEUCINE	100	1.3	1.7	0.19
GLYCINE	100	7.1	8.7	1.3
PROLINE	100	4.1	3.2	0.035
ASPARTIC ACID	100	12.6	15.4	1.1
PHENYLALANINE	100	1.5	2.3	0.01
GLUTAMIC ACID	100	1.2	1.1	0.73

TABLE 5
EXTENT OF RACEMIZATION OF AMINO ACIDS
IN W-1 BASALT
(% D-AMIMO ACID (MEASURED DIRECTLY FROM CHROMATOGRAM))

| | TEMPERATURE °C/TIME (hr) | | | |
	CONTROL	150°/72	200°/24	275°/24
VALINE	1.3	3.5	3.5	2.0
ALANINE	1.9	12.1	–	16.1
ISOLEUCINE	–	–	–	–
LEUCINE	1.8	12.7	4.6	–
PROLINE	2.0	11.8	12.1	–
ASPARTIC ACID	2.4	7.2	–	15.4
PHENYLALANINE	2.5	9.1	6.3	–
GLUTAMIC ACID	2.0	5.4	5.0	16.6

TABLE 6
PERCENT D-AMINO ACIDS IN HEAT-TREATED
MURCHISON METEORITE

TEMPERATURE-TIME	ASPARTIC ACID	GLUTAMIC ACID
CONTROL	10.7	16.1
150-72	37.3	28.4
200-24	36.6	23.7
275-24	–	–

TABLE 7
TOTAL AMINO ACID NITROGEN (µgN/gm SOIL) AS A FUNCTION OF TIME AND TEMPERATURE

TIME (hr)	TEMPERATURE °C			
	125°	150°	200°	275°
24	58.5	–	34.0	-10.0
72	56.0	62.5		
120	51.0	56.0		
CONTROL	53.5			

TABLE 8
TOTAL AMINO ACID NITROGEN (µgN/gm SOIL) AS A FUNCTION OF CHEMICAL STERILIZATION PROCEDURE

CONTROL	GLUTARALDEHYDE DILUTE (2.5%)	GLUTARALDEHYDE CONC. (6%)	OZMIUM TETROXIDE	TRIPLE-FIX
53.5	124	48.5	35.5	28.0

TABLE 9

CONCENTRATIONS OF AMINO ACIDS (NMOLE/gm) IN HEAT AND CHEMICAL STERILIZED SOIL SAMPLES

AMINO ACID	CONTROL	125°/ 24	125°/ 72	125°/ 120	150°/ 72	150°/ 120	200°/ 24	275°/ 24	DIL. GLUT.	CONE GLUT.	OZMIUM TETROX.	TRIPLE FIX
					TEMPERATURE °C/TIME (hr)							
ASPARTIC ACID	324	252	268	258	239	250	217	21.4	818	142	47.4	184
THREONINE	86.9	92.8	30.9	32.6	17.9	25.2	10.8	TR	162	37.3	13.3	34.0
SERINE	132	138	88.3	77.8	70.2	70.5	30.5	3.0	294	90.9	44.4	105
GLUTAMIC ACID	198	226	182.4	192	177	168	207	62.0	655	102	45.2	134
PROLINE	52.9	73.3	—	—	—	55.6	59.3	TR	—	—	—	65.3
GLYCINE	337	332	314	308	341	272	294	27.2	1027	193	95.4	222
ALANINE	.247	278	227	219	402	188	211	16.0	824	156	46.7	177
CYSTEINE	21.5	?	?	—	—	—	—	—	?	—	—	6.3

TR = TRACE

GLUT. = GLUTARADEHYDE

TETROX. = TETROXIDE

TABLE 9 (CONT.)

CONTRATIONS OF AMINO ACIDS (NMOLE/gm)
IN HEAT AND CHEMICAL STERILIZED SOIL SAMPLES (CONTINUED)

AMINO ACID	CONTROL	TEMPERATURE °C/TIME (hr) 125°/24	125°/72	125°/120	150°/72	150°/120	200°/24	275°/24	DIL. GLUT.	CONE GLUT.	OZMIUM TETROX.	TRIPLE FIX
VALINE	80.9	52.4	37.4	–	33.2	–	56.6	3.7	209	7.1	9.4	35.5
METHIONINE	28.2	27.0	29.7	–	28.3	11.7	7.3	–	37.1	6.9	5.0	7.0
ISOLEUCINE	29.9	52.1	51.7	27.6	52.8	34.4	40.3	5.0	336	25.7	16.8	36.7
LEUCINE	63.3	94.0	71.7	87.6	97.6	63.0	68.9	7.1	315	44.7	17.6	66.8
TYROSINE	10.6	7.3	?	?	25.7	–	?	–	?	–	–	TR
PHENYLALANINE	42.3	41.8	38.5	27.7	50.3	31.6	34.9	?	137	–	13.8	26.6
LYSINE	41.6	67.6	55.8	37.6	39.6	33.1	29.3	–	81.4	12.4	10.6	13.3
HISTIDINE	–	12.2	9.4	–	11.6	5.5	–	–	34.0	4.3	–	2.8
ARGININE	34.5	55.1	66.9	45.4	49.4	32.2	12.8	–	125.1	16.0	13.4	19.3
TOTAL	1730.6	1801.6	1471.7	1313.3	1636.1	1240.8	1279.7	145.4	5054.6	838.3	379.0	1135.6

TR = TRACE
GLUT. = GLUTARALDEHYDE
TETROX. = TETROXIDE

TABLE 10

PERCENT D-AMINO ACIDS IN SOIL SAMPLES AS A
FUNCTION OF TEMPERATURE-TIME AND CHEMICAL TREATMENTS

TEMPERATURE °C/TIME (hr)

AMINO ACID	CONTROL	125°/24	125°/72	150°/24	150°/72	150°/120	200°/24	275°/24	DIL. GLUT.	OZMIUM TETROXIDE	TRIPLE FIX
VALINE	1.7	2.2	–	2.5	4.3	5.7	–	47.2	1.2	–	–
ALLOISOLEUCINE/ ISOLEUCINE	0.7	–	–	–	–	4.8	–	28.8	–	–	–
ALANINE	7.9	8.6	9.4	9.8	12.3	12.8	21.2	36.1	6.9	5.0	9.4
LEUCINE	3.2	2.7	3.9	3.6	4.6	5.0	14.6	45.4	3.8	–	9.8
PROLINE	1.0	1.7	2.2	2.9	3.3	4.3	8.8	30.8	2.0	3.6	–
ASPARTIC ACID	8.6	13.6	16.1	22.1	24.2	24.8	33.3	42.5	12.0	10.9	13.5
PHENYLALANINE	2.7	–	2.7	–	2.9	2.6	16.3	47.4	–	–	–
GLUTAMIC ACID	11.5	11.2	13.5	14.2	19.8	19.5	30.3	45.4	11.0	8.8	10.6

FIGURE 1
SCHEME OF ANALYSIS FOR W-1 BASALT AND MURCHISM METEORITE
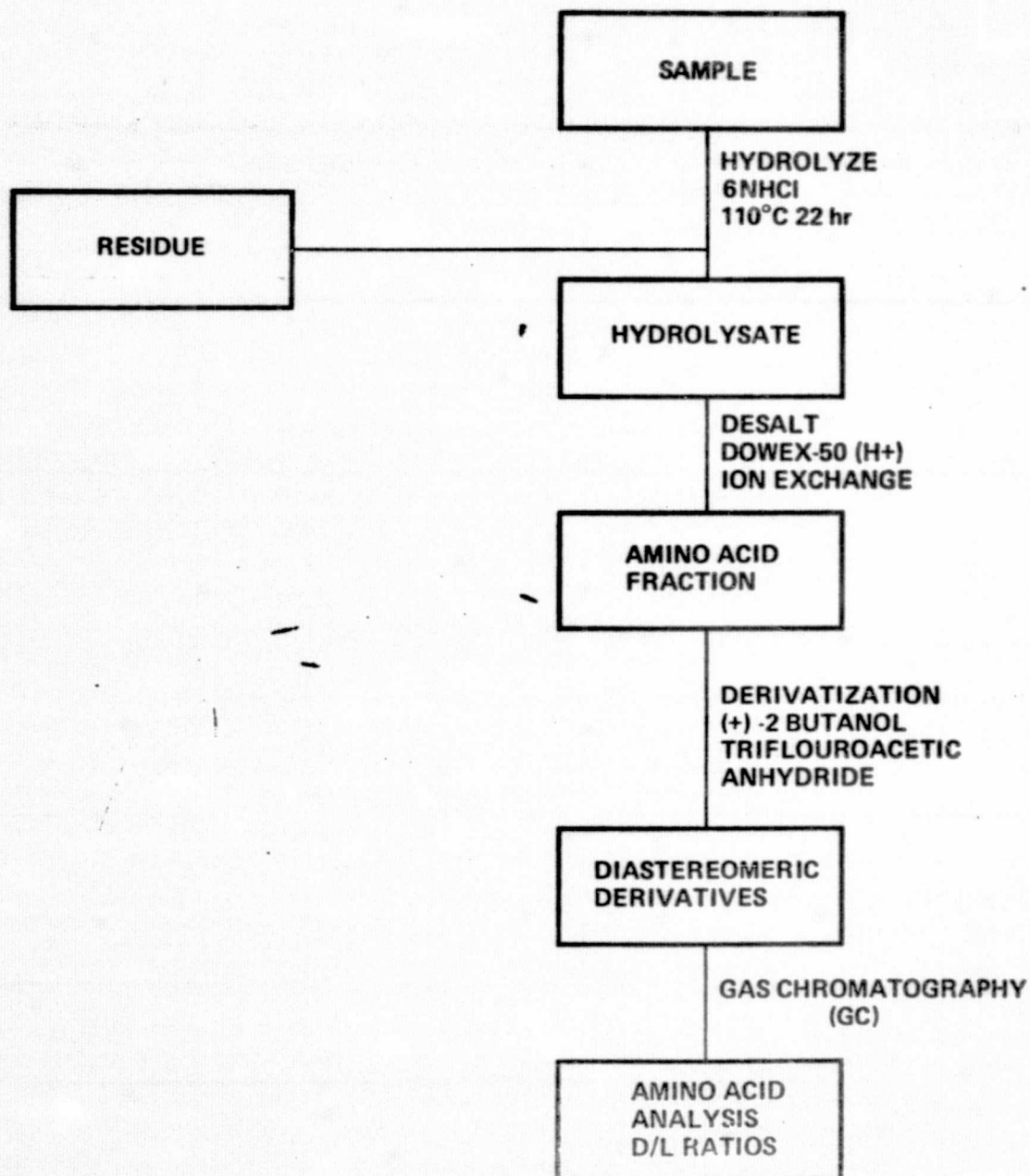
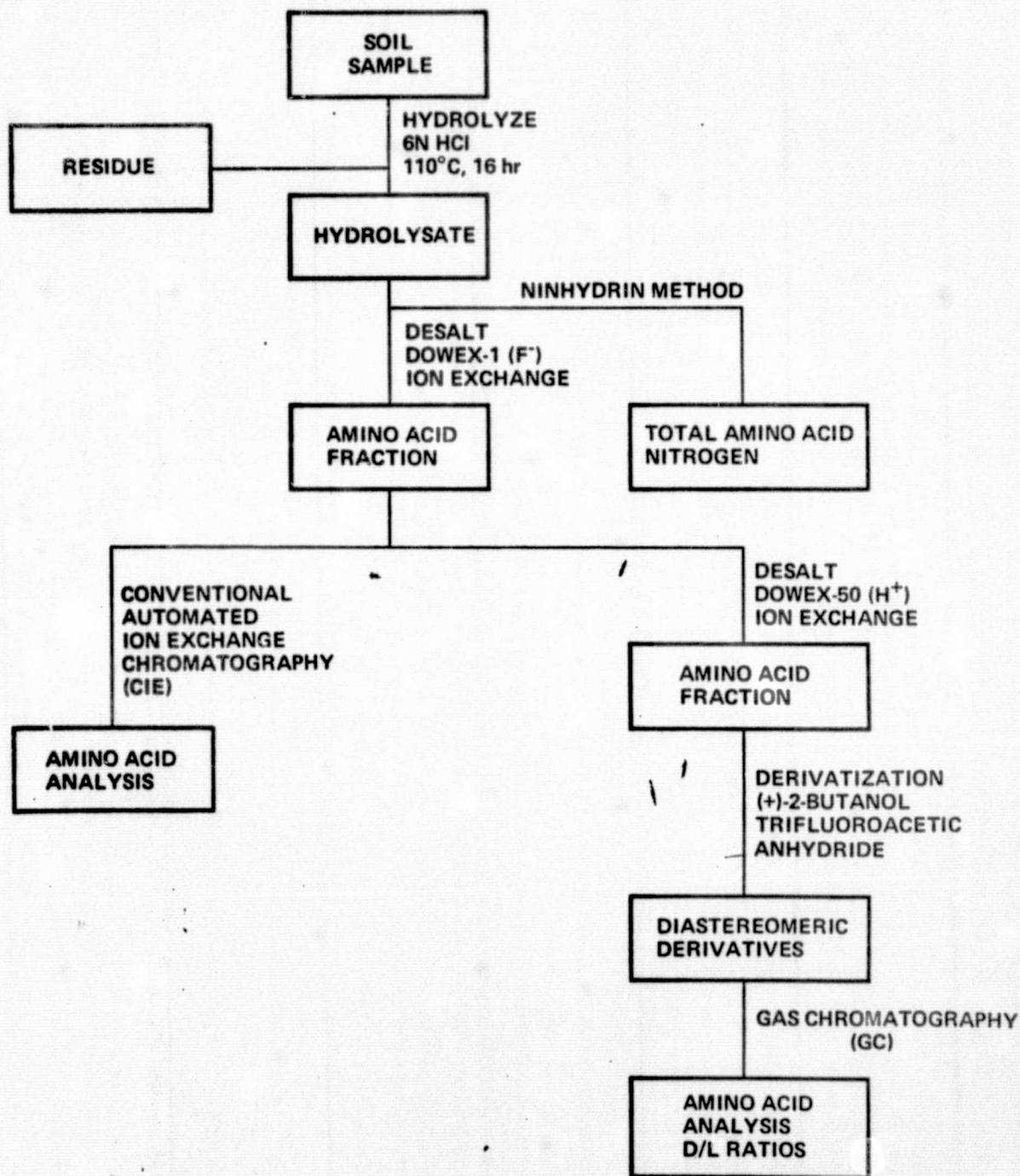# FIGURE 2
SCHEME OF ANALYSIS FOR HERPERIA SOIL

Fig 3

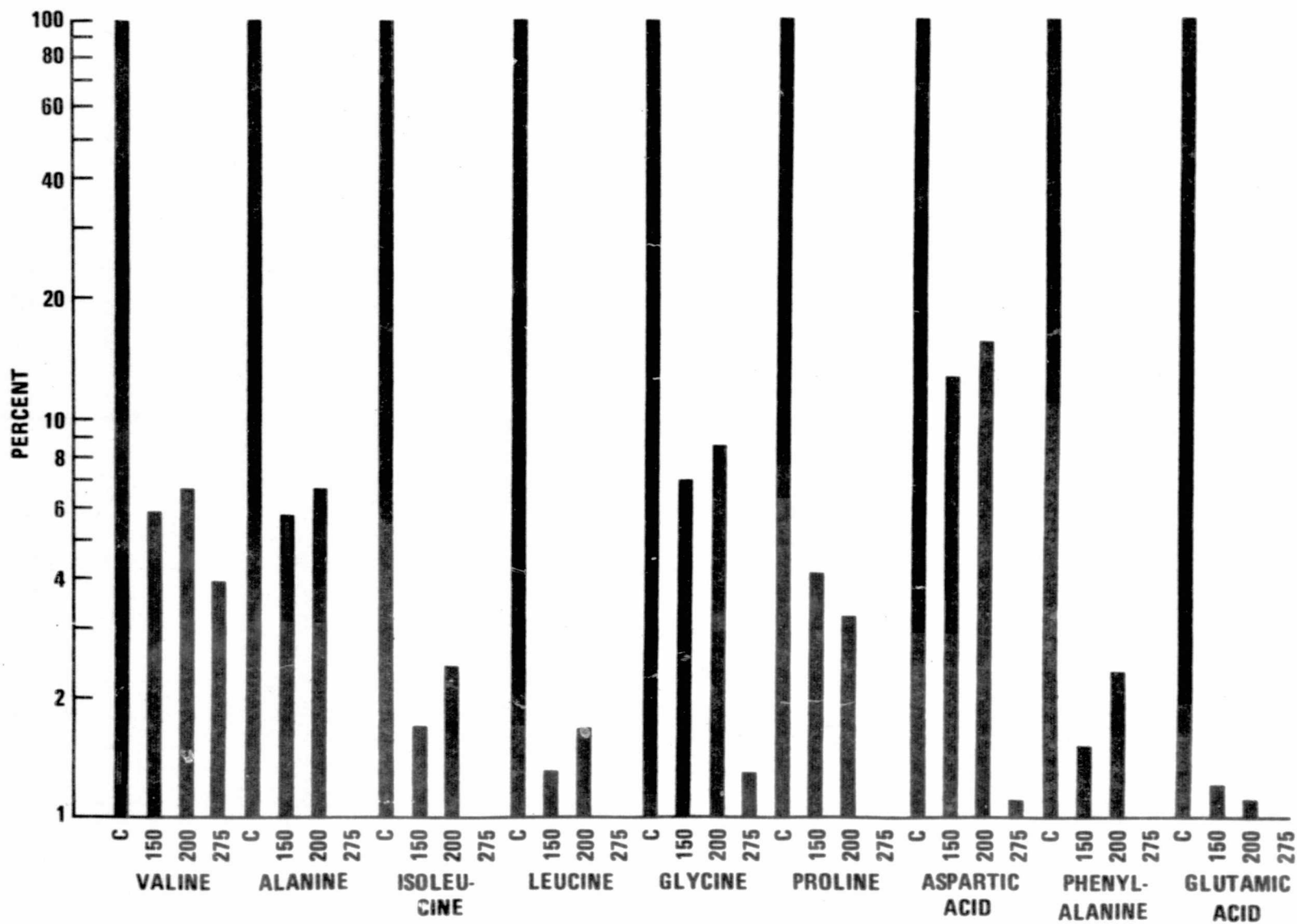PERCENTAGES OF AMINO ACIDS RECOVERED FROM W-1 BASALT
(RELATIVE TO CONTROL)

Figure 4. Gas chromatograms of amino acids from Murchison

Meteorite

Control

Identification: (1) isovaline, (2) α-amino isobutyric

acid, (3-4) D-valine and L-valine + unknown, (5) D-α-amino-n-

butyric acid + D-α-alanine, (6) L-α-alanine + L-α-amino-n-butyric

acid, (7) L-isoleucine, (8) N-methylglycine, (9-10) D and L-

isoleucine?, (11-12) D- and L-β-aminoisobutyric   acid and (DL)-

β-amino-n-butyric acid, (13) glycine, (14-15) D- and L-pipecolic

acid plus contaminants, (16) β-alanine, (17-18) D- and L-proline,

(19) γ-aminobutyric   acid, (20-21) D- and L-aspartic acid, (22-

23) D- and L-phenylalanine, (24-25) D- and L-glutamic acid.

Figure 5. Gas chromatograms of amino acids from Murchison
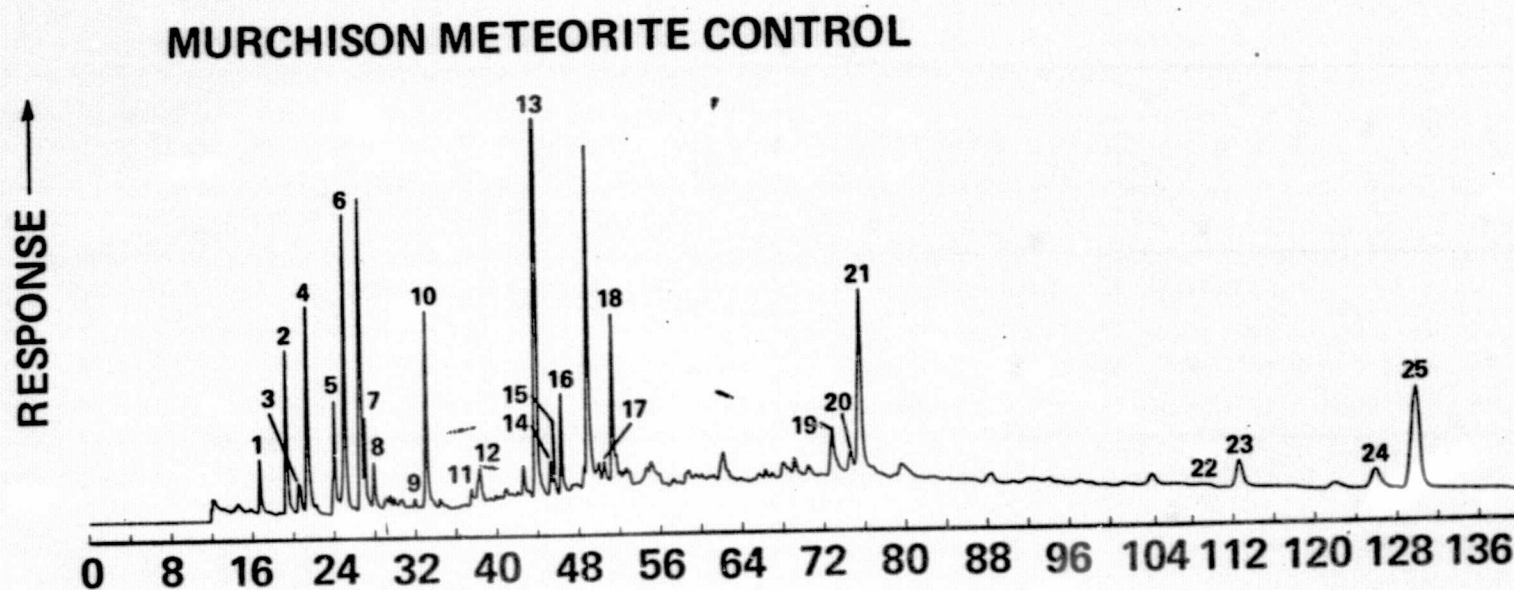
Meteorite

Sample Heated at 275°C for 24 hours.

MURCHISON METEORITE CONTROL
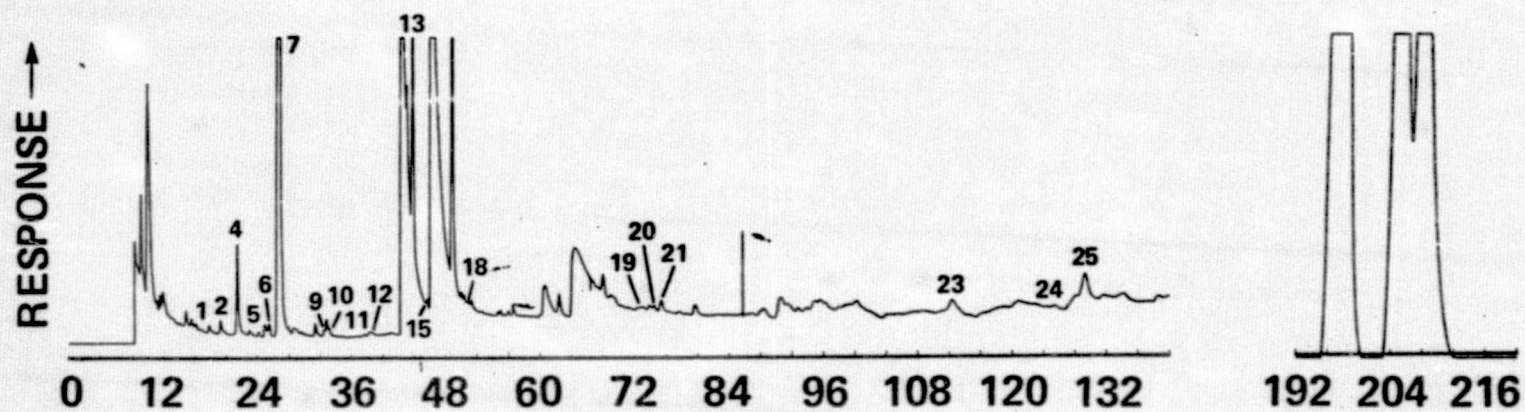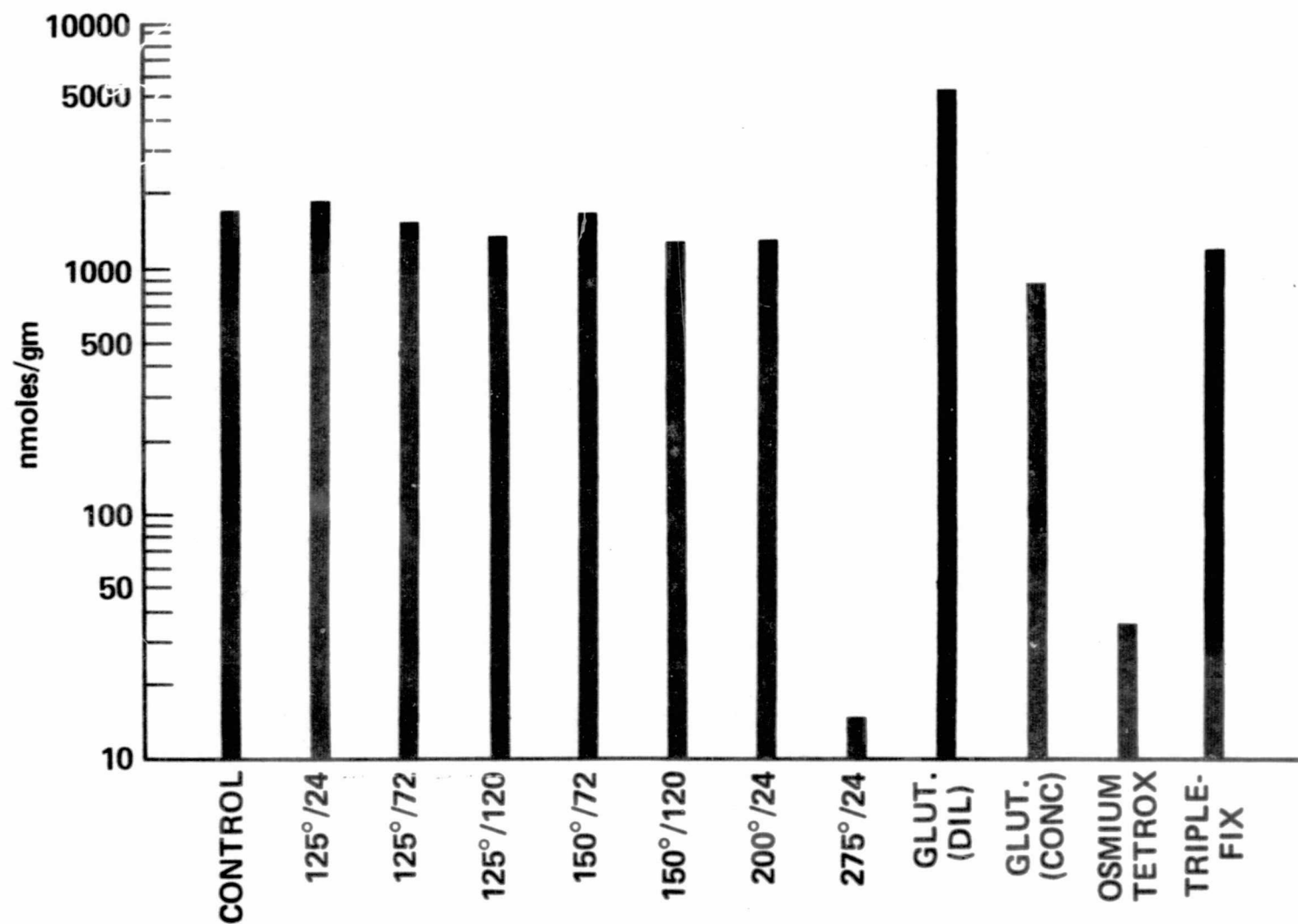
FIGURE 4

MURCHISON METEORITE
275°C — 24 HRS.

FIGURE 5

Fig 6

CONCENTRATION (nmoles/gm) OF TOTAL AMINO ACIDS
IN HEAT AND CHEMICAL STERILIZED SOIL

Figure 7. Gas chromatograms of amino acids from Hesperia soil

Control

Identification: (1-2) D-and L-valine, (3) D-alloisoleucine, (4 and 6) D-and L-alanine, (5) L-isoleucine, (7-8) D-and L-leucine, (9-10) Internal Standard-Norleucine, (11) glycine, (12) β-alanine, (13-14) D- and L-proline, (15-16) D-and L-aspartic acid, (17) γ-aminobutyric acid, (18-19) D-and L-phenylalanine, (20-21) glutamic acid.

Figure 8. Gas chromatograms of amino acids from Hesperia soil

Sample heated at 275 C for 24 hr.

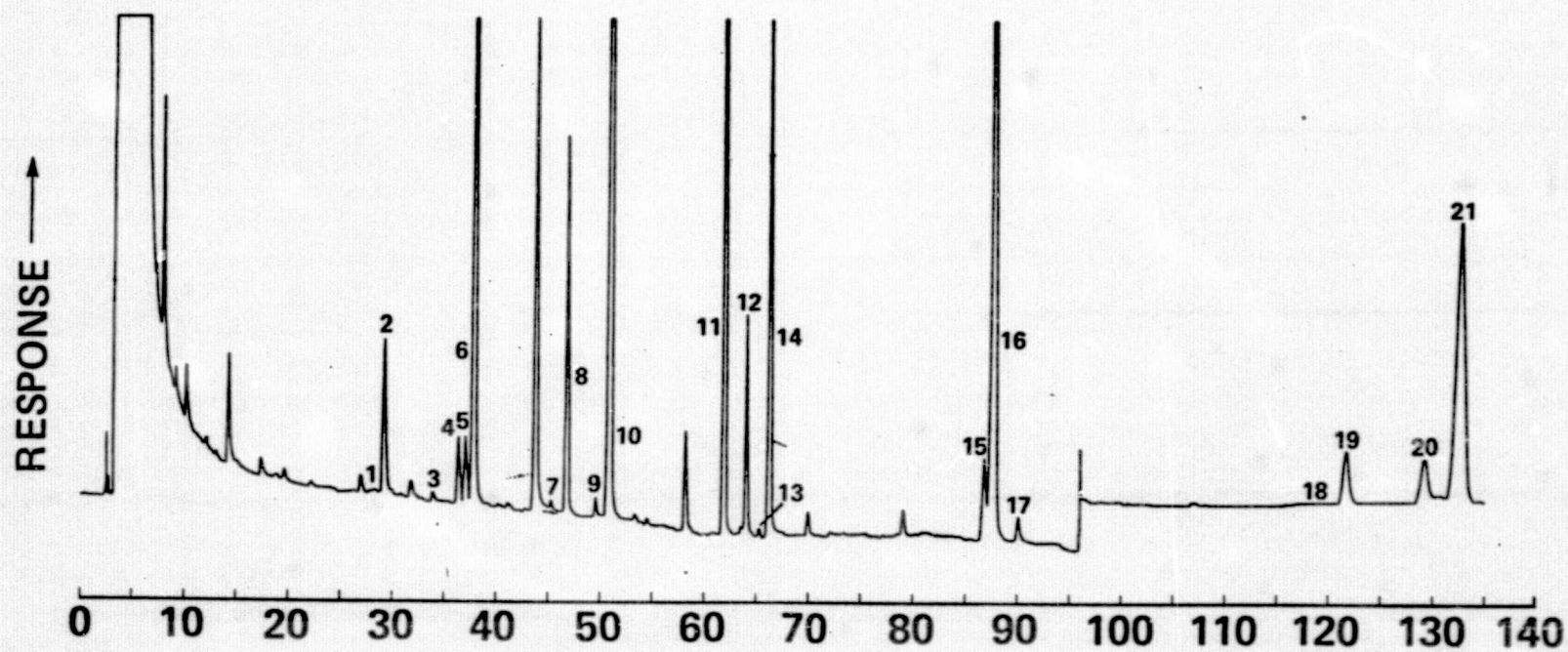Identification of peaks given in legend of Figure 7.
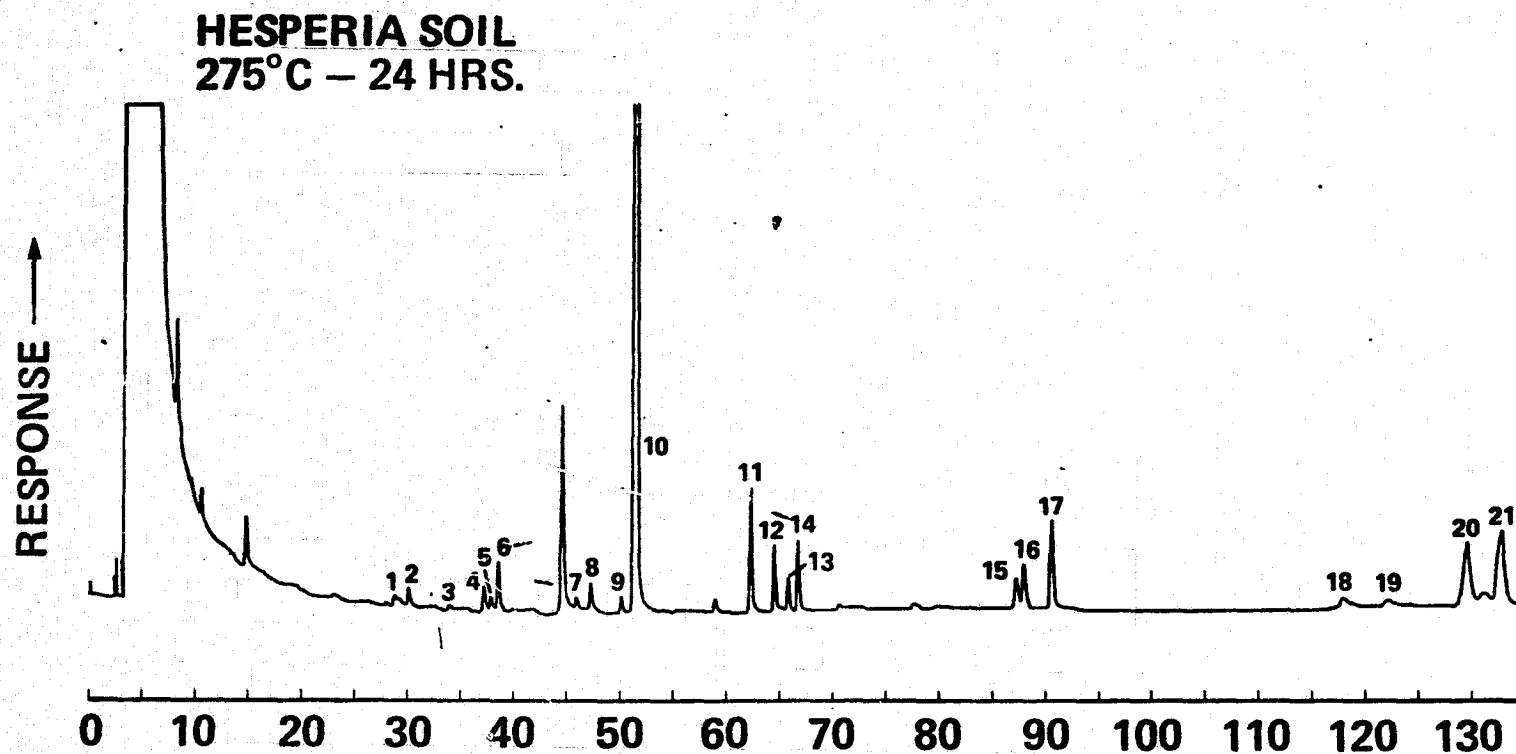
HESPERIA SOIL CONTROL

FIGURE 7

FIGURE 8

EFFECTS OF HEAT AND CHEMICAL STERILIZATION ON THE MORPHOLOGY

OF MICROORGANISMS IN HESPERIA SOIL

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Introduction: The biological impact of finding life forms on Mars is enormous. Such a discovery would mean life has originated independently from Earth and would give clues to its universality and structural organization. Of course, the sample with the most information would be the least treated or altered before examination. The possibility of a back-contamination hazard, however, shows the necessity for finding sterilization procedures that minimally alter the morphology, chemistry, and integrity of the sample. Chemical and heat-sterilization procedures were designed to provide preliminary data and guidelines for future work and to ascertain the chances of success for chemical and/or heat sterilization. While chemical sterilization has the disadvantage of adding material to the sample, it has the advantage of increasing its effectiveness with time. Since fixation occurs by reacting and crosslinking with the biological molecules, the fixative could be left on the sample during its return, thereby assuring sterilization and preservation.

Methods: A standard soil sample (Hesperia) was used to provide uniform data for each treatment. Control samples of 0.1, 0.5, 1.0, 5.0, and 20 gm were examined under light and electron microscopes. Soil was prepared by the following methods:

(1) Untreated soil was placed on EM grids and SEM stubs for direct observation.

(2) Soil was grown up to increase the microorganism content and then placed on EM and SEM holders for observation.

(3) Soil received from the microbiology group was heated at various temperatures and for various periods of time. It was prepared by 5% HF treatment for 24 hr at room temperature and the supernate was spun down by slowly increasing the g forces every 5 min and held at maximum (9700 g) for 15 min.

(4) Soil was grown up, dried over P_2O_5, and then heated to 200°C for 24 hr and examined in the TEM.

(5) Soils were also chemically treated and a ratio of 10 parts fixative to 1 part soil was always used. The organic geochemistry group determined the amino acid content of soils fixed in 6% glutaraldehyde, 1% osmic acid, and triple fix (3% glutaraldehyde, 1% formaldehyde, and 0.5% dinitrodifluorobenzene). Sterility testing of the 6% glutaraldehyde and triple fix was carried out on T. Soy, Sabouraud, and glycerol yeast extract media.

(6) To test the sterilizing capability of the triple fix and to get an idea of possible growth inhibition caused by residual chemicals that might remain after washing, 0.5 gm of fixed, washed soil was added to each of 20 test tubes containing 5 ml of 10% soil extract and 1% Tryptic soy broth (see Fig. 1). These tubes were checked for sterility. 0.5 gm of fixed soil was added to another set of 20 test tubes in the same manner. To this group of tubes, 0.05 gm of untreated soil was added to 10 tubes and 0.025 gm was added to the remaining 10 tubes. Untreated soil was mixed with the treated soil by shaking; these tubes provided the growth inhibition testing. 0.5 gm of autoclaved, washed soil was also added to each of a third set of 20 tubes to be used as turbidity standards.

(7) Microorganisms were extracted and concentrated by several methods. Pellets of material for study were produced by brief sonication, shaking, in pyrophosphate and in 5% H.F., to loosen the microorganisms and concentration by centrifugation.

Results: Hesperia soil was examined by light, electron, and scanning electron microscopy. The microorganisms were extracted from untreated, heat treated, and chemically treated aliquots. The microorganisms were removed from the soil by sonication and shaking and by 5% HF and pyrophosphate treatment. However, sonication was very short in order to remove but not disrupt the organisms. It was also found that 5% HF dissolved 60% of the soil, freeing many, if not all, of the microorganisms. Some of the resulting extract was plated out on three types of media, T. Soy, yeast extract, and Sabouraud dextrose. These media were also poured over the samples. Excellent growth resulted (see pictures) and this allowed examination of the viable organisms in the soil.

Heat-sterilization work (L. Hochstein) indicated the 150°C for 24 hr and 200°C for 24 hr sterilizations as the important samples to examine. Consequently, work at lower temperatures was stopped and the 150°C and 200°C samples were examined. While 7×10^6 microorganisms/gm were predicted, our sample of 0.1 gm yielded few organisms per field in the transmission electron microscope from negative stained and unstained grids. Nevertheless, the dried shapes of rods and spheres seen were considerably more flattened than the non-heat-treated material. The increased flattening from the surface tension during drying indicates a possible breakdown of internal structure. X-rayed bacteria and virus (Philpott, unpublished) also produces increased flattening upon drying.

Because of the difficulty in finding organisms in the 150°C, 24-hr sample, we grew up a larger sample to increase the number of microorganisms, then dried and heated it at 200°C for 24 hr. Again, flattened cells appeared along with many unidentifiable shapes. The cells had ballooned out areas and appeared more disrupted than those heated to 150°C for 24 hr. Our preliminary observations indicate a decrease in organized structure as the temperature increases. Further testing with larger samples is necessary.

Chemical sterilization and preservation were performed using 6% glutaraldehyde, 1% osmium and a triple fix (consisting of glutaraldehyde, formaldehyde, and dinitrodifluorobenzene), which has been shown to provide excellent prolonged preservation of ultrastructure for up to 2 yr (see attached publication). Streaking 6% glutaraldehyde fixed soil on agar after four sterile washes and pour plating on three basic media gave no growth. All of our plating and pouring (standard media only) gave sterile results with the triple fix. Since this fixative has small molecules that penetrate cells, it has proved to be a good spore fixative. An experiment was set up to test its sterilizing capabilities (see Fig. 1). Two sets of tubes were also seeded with untreated soil to check on possible residual fixative that might inhibit the growth of the microorganisms. All of the seeded tubes grew while none of the controls grew, indicating there was no chemical inhibition for these experimental conditions.
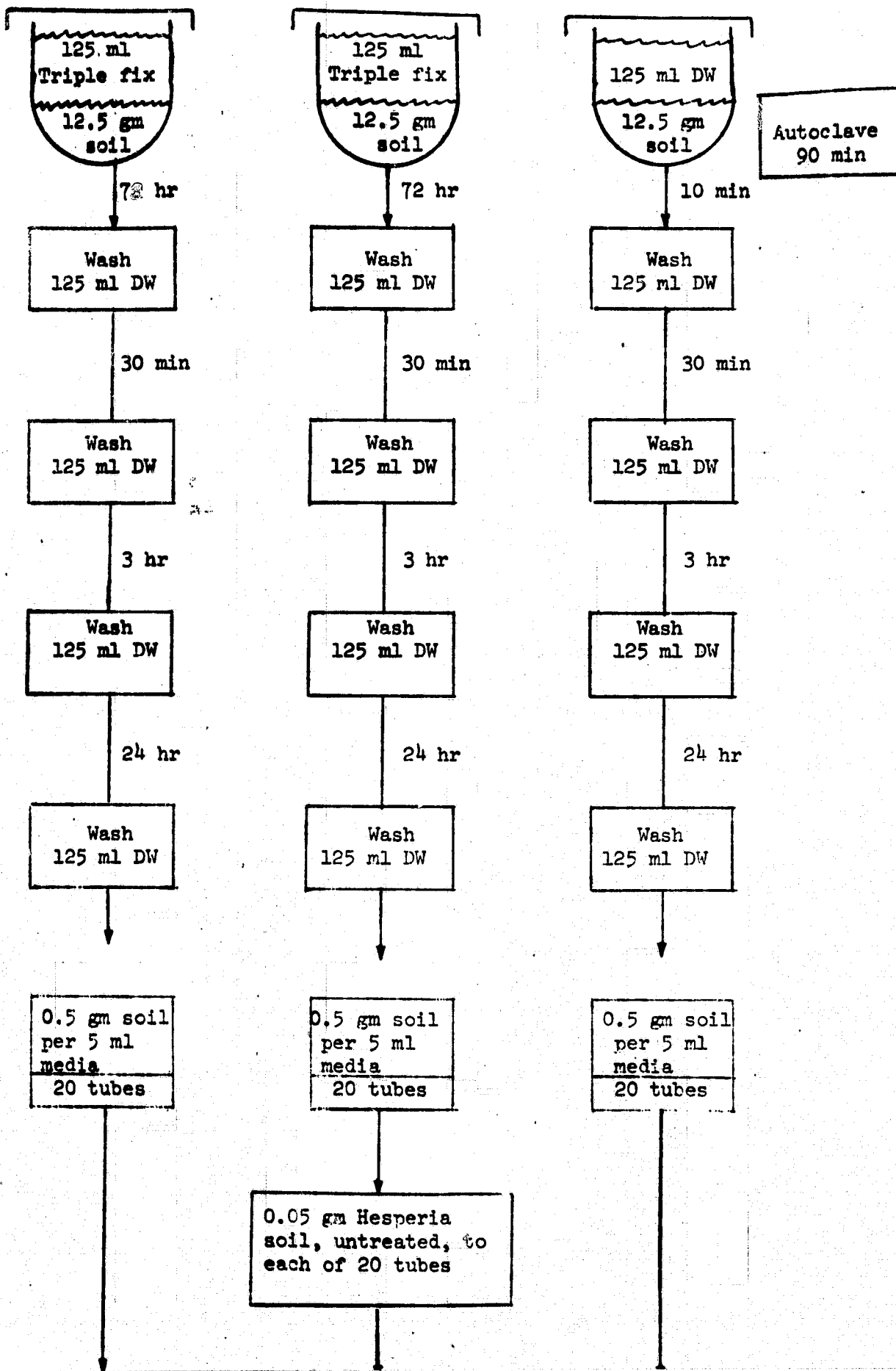
The amino nitrogen doubled in a 2-1/2% glutaraldehyde sample from the Hochstein laboratory, indicating growth; they remained the same in 6% glutaraldehyde. Osmium decreased the amino nitrogen level by 50% while triple fixation decreased the amino acid level by 40% (G. Pollock).

While freeze fracture can reveal internal cellular detail, it has serious limitations for a Mars sample return. At least 90% of the sample used for freeze fracture is lost on each run. The problems involved with getting a pellet ready to freeze fracture are the same as getting it ready for other methods, i.e., sectioning, shadowing, scanning, and X-ray fluorescence probing. However, these other methods do not waste the pellet. Hence this method was reserved for later testing.

Conclusions: During the short time available, only the most necessary problems could receive attention and even these could not be completely worked out. It appears, with these preliminary runs, that heat and chemical sterility can be achieved and that gross structural alterations occur at 200°C for 24 hr and that alterations also occur at 150°C. The degree of alteration may be determined after more electron micrographs can be obtained and more samples run. Little or no morphological alterations occur with glutaraldehyde or triple fix sterilization. While identification of microorganisms can be made by viewing the surface morphology, a more positive identification can be made with thin sectioning to observe the internal ultrastructure; this would be a most exciting way to view Mars biota.

Recommendations:

(1) Develop more effective procedures to concentrate the microorganisms.
(2) Heat the soil while it is in the fixative to ascertain its effect on sterilization and morphology.
(3) Develop techniques for analyzing soils for residual chemical fixatives.
(4) Further study the use of the electron dispersive analysis X-ray system (EDAX) for identifying microorganisms.
(5) Pulse heat the sample to determine the effectiveness of such a method.

125 ml Triple fix / 12.5 gm soil	125 ml Triple fix / 12.5 gm soil	125 ml DW / 12.5 gm soil	Autoclave 90 min
↓ 72 hr	↓ 72 hr	↓ 10 min	
Wash 125 ml DW	Wash 125 ml DW	Wash 125 ml DW	
↓ 30 min	↓ 30 min	↓ 30 min	
Wash 125 ml DW	Wash 125 ml DW	Wash 125 ml DW	
↓ 3 hr	↓ 3 hr	↓ 3 hr	
Wash 125 ml DW	Wash 125 ml DW	Wash 125 ml DW	
↓ 24 hr	↓ 24 hr	↓ 24 hr	
Wash 125 ml DW	Wash 125 ml DW	Wash 125 ml DW	
↓	↓	↓	
0.5 gm soil per 5 ml media / 20 tubes	0.5 gm soil per 5 ml media / 20 tubes	0.5 gm soil per 5 ml media / 20 tubes	
	↓		
	0.05 gm Hesperia soil, untreated, to each of 20 tubes		

Appendix: Soil Sterilization - Combined effects of heat and radiation

To determine if radiation in combination with heat would lower the temperature required to sterilize soil, we have initiated a preliminary set of experiments as an addendum to the recent dry heat experiments conducted by members of the Biological Adaptation Branch at Ames. The purpose of such experiments is to minimize the thermal destruction of biological, chemical, and geological material in soil.

We used "Hesperia" soil in these experiments; the same as that used for the B.A.B. dry heat experiments. This soil contained 5.8×10^6 aerobic organisms per gram of which about 10% were spore-formers. Approximately 100 mg samples of soil were placed in each of 40 glass tubes (13 x 1.6 cm) and cotton plugs were inserted. One half of these tubes were autoclaved (121°C, 15 psi) for 90 min and constitute "sterile" controls. All 40 tubes were placed in a 1500 ml beaker and represent one experimental set. Ten such sets were prepared and exposed to heat and/or radiation as indicated in Table I. The sets which were heated were exposed, during the heating cycle, to a continuous vacuum of 10-15 mm mercury. Radiation doses were administered with a ^{60}Co Gamma cell within two hours of the heat exposure. Following heat and/or radiation exposure, each tube was filled with 5 ml of sterile 1% Tryptic Soy Broth supplemented with 10% soil extract. All tubes were incubated

at 25°C and observed for turbidity at 7 day intervals for 3 weeks. If a tube was scored turbid, the cause of the turbidity was determined microscopically and in all such cases the turbidity was traced to easily identifiable, numerous, bacterial forms (primarily rod-shaped organisms). Once scored as a positive and microbes were observed microsopically, the tube was discarded. At the end of three weeks all tubes lacking turbidity were scored as negatives and discarded.

Our results, given in Table II, indicate that 123°C or 137°C dry heat for approximately 20 hr was insufficient to sterilize 100 mg soil samples. These results are in agreement with the previous dry heat study conducted by B.A.B. If the heat cycle was followed by a radiation dose of 0.5 Mrad or 1.0 Mrad, the number of positive tubes was reduced to zero. When the same two doses were applied to unheated soil samples, all tubes became turbid. Thus, these doses will not sterilize unheated soil.

Radiation doses below 0.5 Mrad when coupled with a dry heat cycle of 20 hr at 137°C gave inconclusive results. It seems reasonable however, to expect an inverse relationship between heat and radiation exposures which, when coupled, will sterilize soil.

In summary, soil can be rendered sterile (does not yield turbid broth cultures) by non-sterilizing dry heat exposures, if the heat cycle is followed by an exposure to a non-sterilizing dose of gamma radiation. Whether the increase in biologically, chemically, and geologically useful information gained by reducing thermal destruction is offset by radiation damage remains to be determined.

TABLE I

EXPERIMENTAL CONDITIONS

HEAT / RADIATION[2]	25°C [1]	123°C	137°C
0	ND [3]	+	+
0.01	+	ND	+
0.10	+	ND	+
0.50	ND	+	ND
1.00	+	+	+

1. Sets not subjected to heat, kept at room temperature during experiment.
2. Radiation doses x 10^6 rad.
3. ND - Not determined.

TABLE II

SAMPLES YIELDING TURBIDITY FOLLOWING EXPOSURE TO HEAT AND/OR RADIATION [1]

HEAT / RADIATION[2]	25°C		123°C [3]		137°C	
	EXPTL	CONTL	EXPTL	CONTL	EXPTL	CONTL
0	ND [4]		19	0	12	0
0.01	20	0	ND		19	0
0.10	20	0	ND		10	2
0.50	ND		0	0	ND	
1.00	20	0	0	0	0	0

1. In all cases the sample size is 20 tubes therefore only the numerator of the fraction positive is given.
2. Radiation doses x 10^6 rad.
3. Observations after 2 weeks.
4. ND - Not determined.