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FINAL REPORT

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THE GENERAL AND COMPARATIVE BIOLOGY OF TERRESTRIAL ORGANISMS UNDER EXPERIMENTAL STRESS CONDITIONS

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TABLE OF CONTENTS

I. INTRODUCTION

This volume constitutes the final report under contract NASw-767. Additional work carried out before the end of the fiscal year, 29 June, 1967, will be integrated with any continuation program report under grant sponsorship at the University of Hawaii.

Each section of the present report represents a continuation of research subjects discussed in the report of 1 November 1966 (and previous reports). The main themes deal with altered liquid media, toxicants and noble gas anoxia. Some of the material, such as section II A which is concerned only with the demonstrated phenomenon of life processes in the most hypertonic aqueous liquid possible, saturated LiCL, is purely descriptive. In contrast, quantative data in relation to KCl suppression of growth in Penicillium notatum (strain UC/SAL-2) is presented--a decided advance in our knowledge of mechanisms of stress resistance.

The most novel subjects deal with the same Penicillium mutant grown with boron and heavy metals and other elements in the nutrient media. The results justify, we feel, the designation of a subject area, "Bio-Inorganic Chemistry," as a new look at biochemical interactions with the elements and their simple derivatives outside the conventional limits prescribed by inorganic nutrition. These is also, a geobiochemical facet to this work.

In the last report, we claimed Penicillium spore germination in a liquid NH_3 medium at 233°K. The evidence provided by tritium-label incorporation studies now verifies at the biochemical level our optical and electron microscopic evidence at spore germination.

Other subjects include continuation of our work in noble gas anoxia and study of heme enzymes activity in aqueous NH_3.

II. ULTRA-SALINE MEDIA

A. Further Observations upon Microflora in Saturated LiCl Nutrient Media

A bacterial mutant resembling Bacillus megaterium was isolated from LiCl-saturated nutrient broth cultures after several months' incubation with Penicillium spores. This distribution of the Bacillus suggested that it was derived from random fallout of airborne (contaminant) spores. More recently, some 32 cultures of Li-saturated glucose-peptone-yeast broth were examined after one year's incubation at 25°C. Most of these cultures were inoculated originally with spores of salt-tolerant Penicillium, but germination was not at all in evidence after several months. Cultures were continued, however, with periodic examination for microbial activity. After a year in saturated Li-acetate, bacteria (especially B. megaterium) were seen in 5 cultures out of 12, and mycelial growths in 3 cultures (Table 1). In the LiCl medium, 10 cultures out of 20 contained bacteria (Table 2). One culture contained yeast-like budding cells, and 3 contained mycelial forms. In addition to the more common, short mycelial clusters, an extended septate hypha was observed (Figure 1). Besides typical bacilloid and coccoid forms, a few unusual structures were seen in one culture (Figure 2). These structures were gram positive non-motile bodies of more or less elliptical form.

Three cultures contained saturating amounts of strontium chloride, magnesium chloride, and calcium chloride in addition to LiCl. These three gave no evidence of microbial activity after one year.

The saturated LiCl + LiOAc cultures were diluted tenfold with nutrient agar and incubated at 25°C for 10 days. During the incubation

period, 8 LiOAc cultures, subcultured from numbers 1, 3, 5-8, 10 and 12 (Table 1) developed typical growth of P. notatum. Four LiOAc cultures, diluted from vessels 6, 7, 10 and 11 produced bacterial colonies and three cultures 2, 4 and 9 appeared totally devoid of life. From the LiCl series, P. notatum was grown in agar incubated with cultures 1, 2, 3-8, and 15-19 (Table 2). Bacteria appeared only in cultures 3, 7 and 11 and cultures 9, 10, 12, 13, 15 and 20 yielded no signs of life.

It seems obvious that mutations toward enhanced osmotic tolerance are far from uncommon.

Sporulation has no such clear cut trends, and seems to involve secondary factors accruing from primary salt injury. Release of amylase also follows a different pattern in detail, even if it is grossly correlated with growth.

Diagrammatically, these findings might well be summarized as follows:

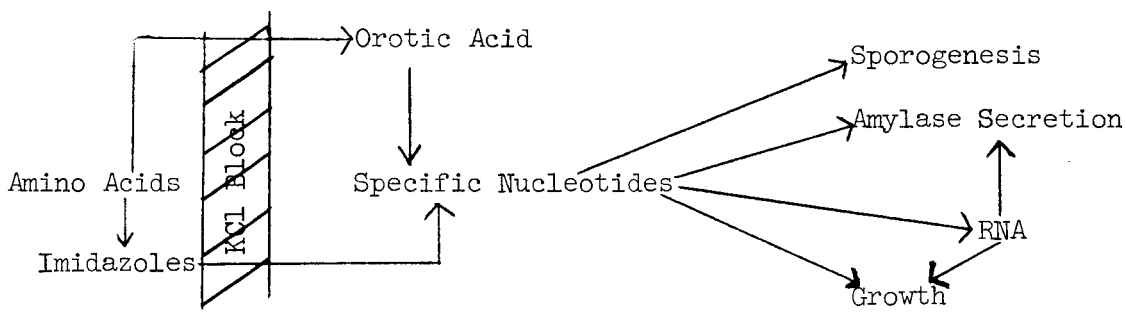


Table 1. Microflora of Year Old Saturated Lithium Acetate in Glucose-Peptone-Yeast Medium*

Vessel	Microflora			
	Hyphae	Yeasts	Cocci	Bacilli
1	+	-	+	+
2	+	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	-	-	+	-
8	+	-	+	+
9	-	-	-	+
10	-	-	-	-
11	-	-	-	-
12	-	-	-	+

*Glucose, 5g/l; peptone, 1g/l; yeast extract, 1g/l.

Table 2. Microflora of Year Old Saturated Lithium Chloride in Glucose-Peptone-Yeast Medium*

Vessel	Microflora			
	Hyphae	Yeasts	Cocci	Bacilli
1	+	-	-	-
2	+	+	+	+
3	-	-	-	+
4	-	-	-	+
5	-	-	-	-
6	+	-	-	-
7	-	-	+	+
8	-	-	-	+
9	-	-	-	+
10	-	-	-	-
11	-	-	-	-
12	-	-	-	+
13	-	-	-	+
14	-	-	-	+
15	-	-	+	-
16	-	-	-	-
17	-	-	-	-
18(+$SrCl_2$)	-	-	-	-
19(+$MgCl_2$)	-	-	-	-
20(+$CaCl_2$)	-	-	-	-

*Glucose, 5g/l; peptone, 1g/l; yeast extract, 1g/l.

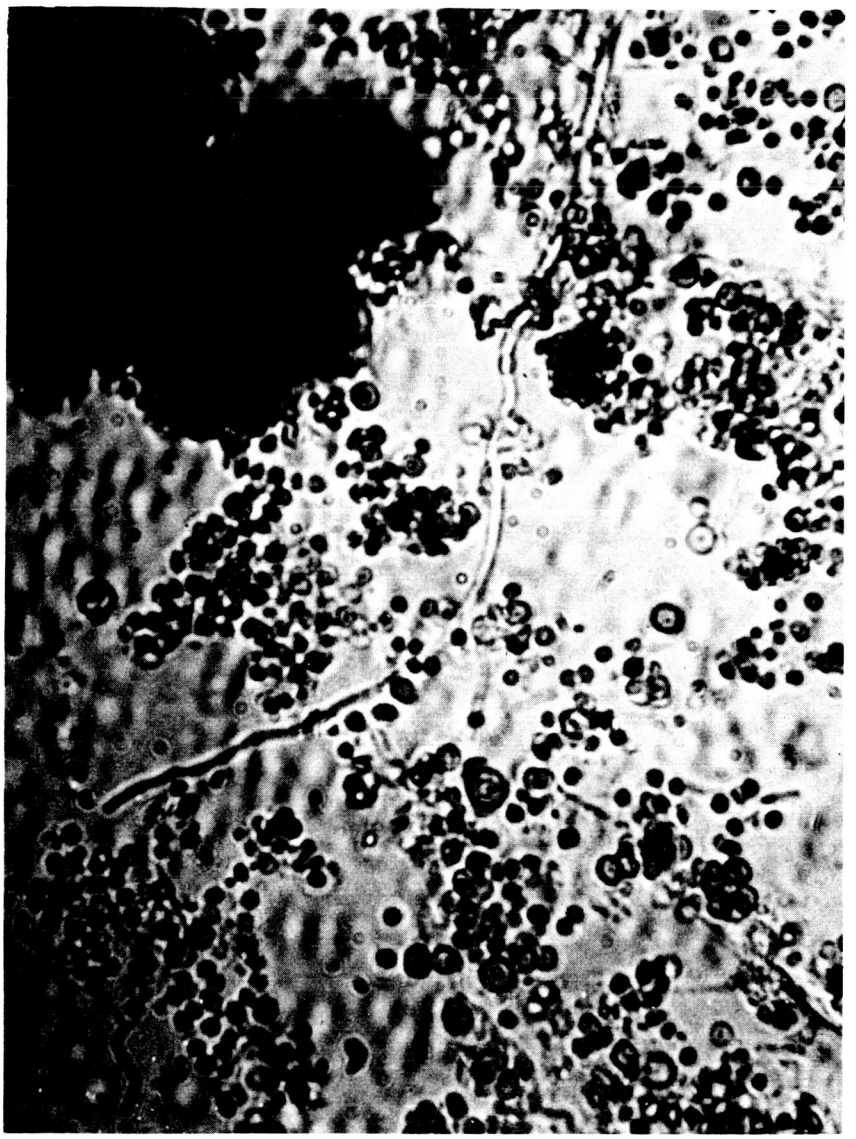


Figure 1 Extended hyphal growth in saturated LiCl nutrient medium inoculated with spores of P. notatum.

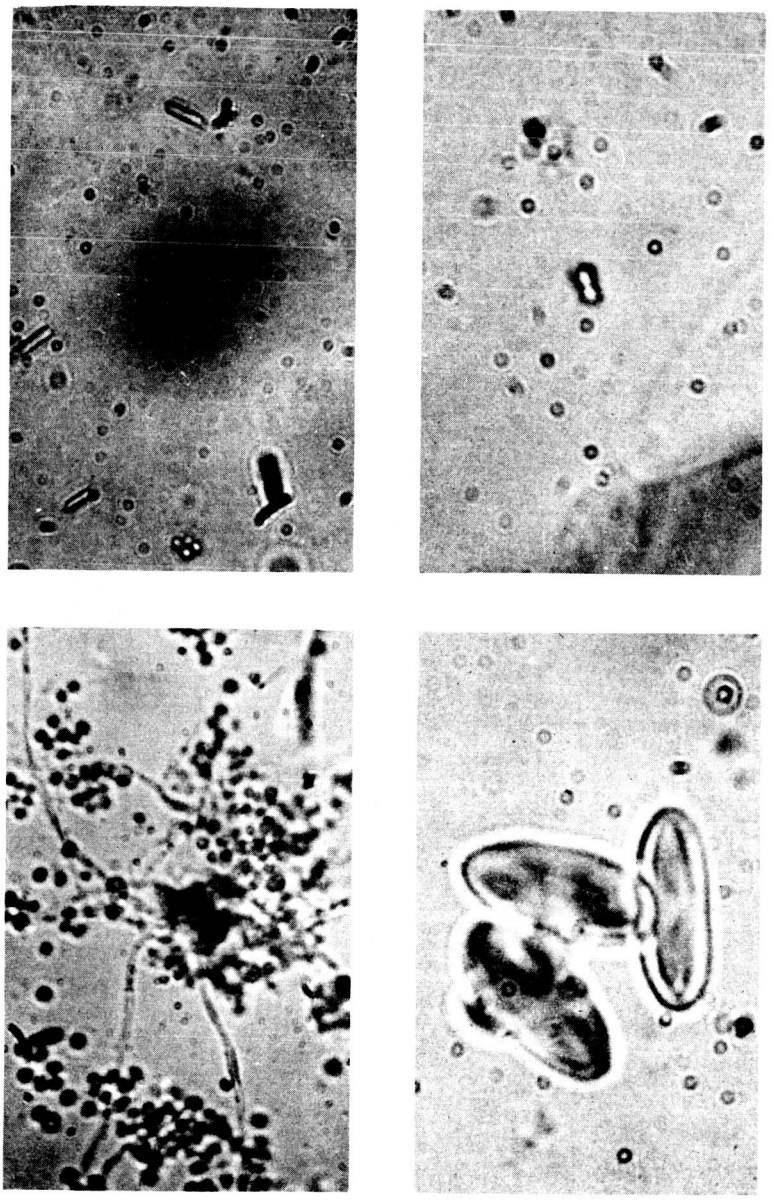


Figure 2 Examples of microbial forms seen in saturated Li salt media (950X).

Upper Left: Motile rod.

Upper Right: Diplococcoid form.

Lower Left: Hyphal cluster.

Lower Right: Unidentified elliptical forms.

B. New Observations On KCl-Habituated Penicillium notatum mutant

The importance of exogenous nucleotides in restoring the growth rate of our calcium acetate mutant after KCl suppression has been established. The alternative pathways suggested for the nucleotide effect were energy metabolism, cell wall synthesis and RNA metabolism. Before examining this system for disturbed metabolic or biosynthetic pathways, other compounds were tested for their ability to effect reversion. These compounds were drawn from several nucleotide sources. These included polymers, special sources such as flavine adenine dinucleotide (FAD) or thiouacil, and precursors (including amino acids). Sporulation as well as growth was noted. Another feature of the KCl-suppressed mutant is its inability to utilize starch as a carbon source. Thus this strain either does not synthesize the required amyloclastic enzymes or can no longer secrete them into its mileau. Using starch agar, hydrolytic zones were observed when IKI solution was added after 16 hr incubation of 9mm mycelial plugs. The general relation between ability of compounds to stimulate growth and their ability to increase the area of the starch-free zone was also examined.

Although heteropolynucleotides show the restorative effect originally observed with yeast extract, E. coli S-RNA was marginally active (Table 3). The two homopolynucleotides tested gave responses opposite to the expected. The previous growth restoring performances of adenine, cytosine and guanine ribonucleotides and the poor performance of UMP have been confirmed; hence it is surprising to find poly A has low activity while poly U stimulates quite well. Although all of the phosphate derivatives of cytosine and guanosine were comparably (and highly) active,

ATP is inferior to the lower adenosine phosphates. In contrast, the uridine derivatives were distinctive in the high performance of UDP only. Some of these differences may relate to permeability, but otherwise, distinctive metabolic fates of closely related nucleoside phosphates may be indicated.

Although dimethyluracil was inactive as expected, thiouracil was a relatively good stimulant. Conceivably its effect relates to the SH function, not to a role in uridine metabolism.

Previously, neither adenine nor adenosine were found to serve in place of AMP. It is not surprising that combined forms of adenosine or AMP can also serve as stimulants. Whether by virtue of selective penetration or degradation, FAD is by far the best growth stimulant. Possibly, FAD functions per se as co-dehydrogenase and not at all as a source of AMP.

Four compounds involved in the early steps of nucleotide synthesis, alanine, aspartic acid, glycinamide and ureidosuccinic, elicited no response from the KCl-habituated fungus. The imidazole intermediates failed to stimulate growth, but orotic acid promoted an appreciable growth response.

Of the seven polymers making up group a, five increased growth to at least 50% of the RNA response, whereas only three promoted soporulation. See Table 3. Among the twelve members of group b, nine enhanced growth substantially, and of this group, only two lacked sporogenic activity. In group c, wherein only FAD stimulated growth, every member stimulated sporogenesis. No member of group d, including orotic acid, elevated the growth rate to specified level, but four compounds were sporogenic, namely the amino acids, ureidosuccinic acid, and orotic acid.

Table 3. Growth of KCl-Habituated *P. notatum* in Salt-Free Glucose-Peptone Media with Various Nucleotide Sources and Precursors

Additions	Growth Rate* mg fr wt/day
None	23 ± 7
a. Polynucleotide Sources	
Yeast Extract	213 ± 16
Yeast RNA	225 ± 14
Yeast S-RNA	180 ± 14
E. coli S-RNA	51 ± 4
Poly Uridylic Acid	120 ± 9
Poly Adenylic Acid	57 ± 5
Protamine nucleinate	158 ± 9
Protamine sulfate	37 ± 4
b. Regular Nucleotide Sources	
AMP	120 ± 11
ADP	129 ± 11
ATP	70 ± 6
CMP	135 ± 12
CDP	143 ± 14
CTP	168 ± 13
GMP	130 ± 10
GDP	125 ± 12
GTP	148 ± 16
UMP	60 ± 8
UDP	107 ± 11
UTP	70 ± 10
c. Miscellaneous Nucleotide Sources	
1,3-Dimethyluracil	25 ± 2
Thiouracil	91 ± 7
Isopropylidene adenosine	36 ± 3
Nicotinamide	52 ± 5
NAD	67 ± 7
FAD	151 ± 13

d. Precursors and Amino Acids

DL-Alanine	23 ± 3
L-Aspartic Acid	39 ± 3
Glycinamide	20 ± 3
Ureidosuccinic	21 ± 5
4-Amino-5-imidazole	39 ± 4
4-Amino-5-imidazole ribonucleoside	38 ± 4
4-Amino-5-imidazole carboxamide	48 ± 5
4-Amino-5-imidazole carboxamide ribonucleoside	28 ± 3
Orotic acid	96 ± 8

*mean of triplicate determinations

There is a correlation between the ability of compounds to promote starch hydrolysis, and their growth-restoring activities (Figure 3). The scatter in this correlation is considerable, and permits only the most general sort of trend to be accepted as valid.

The most active hydrolysis-promoting compounds, aside from yeast extract and whole yeast RNA, were FAD, NAD, ATP, CTP, L-aspartic acid and orotic acid. Dimethyluracil, thiouracil, UTP, DL-alanine and poly A were inactive.

In assessing the responses of KCl-habituated Penicillium to these 35 exogenous factors, it is obvious that mycelial growth, sporulation and amyloclastic activities each require unique treatment. Growth is the most consistent response. It seems reasonable, subject to a great deal of supporting evidence not yet available, to postulate that (1) there is a low-level, non-specific stimulating effect which may result from alleviation of secondary disturbances in growth-dependent mechanisms, (2) high level growth-promotion (or reversion) involves the overcoming or by-passing of blocks in ribonucleotide synthesis at the level of the cyclic precursors. The pathway via orotic acid is obviously not sufficient alone to account for the salt-induced "lesion" in the metabolic system.

Table 4. Sporulation of KCl-Habituated *P. notatum* in Salt-Free Glucose-Peptone Media with Various Nucleotide Sources and Precursors

Additions	Sporulation after 5 days
None	-
a. Polynucleotide Sources	
Yeast Extract	+++
Yeast RNA	+++
Yeast S-RNA	-
E. coli S-RNA	-
Poly Uridylic Acid	-
Poly Adenylic Acid	-
Protamine nucleinate	+++
Protamine sulfate	-
b. Regular Nucleotide Sources	
AMP	++
ADP	++
ATP	++
CMP	+
CDP	++
CTP	++
GMP	++
GDP	++
GTP	++
UMP	-
UDP	-
UTP	+
c. Miscellaneous Nucleotide Sources	
1,3-Dimethyluracil	+
Thiouracil	+
Isopropylidene adenosine	+
Nicotinamide	+
NAD	+
FAD	+

d. Precursors and Amino Acids	
DL-Alanine	+
L-Aspartic Acid	+
Glycinamide	+
Ureidosuccinic	-
4-Amino-5-imidazole	-
4-Amino-5-imidazole ribonucleoside	-
4-Amino-5-imidazole carboxamide	-
4-Amino-5-imidazole carboxamide ribonucleoside	-
Orotic acid	+

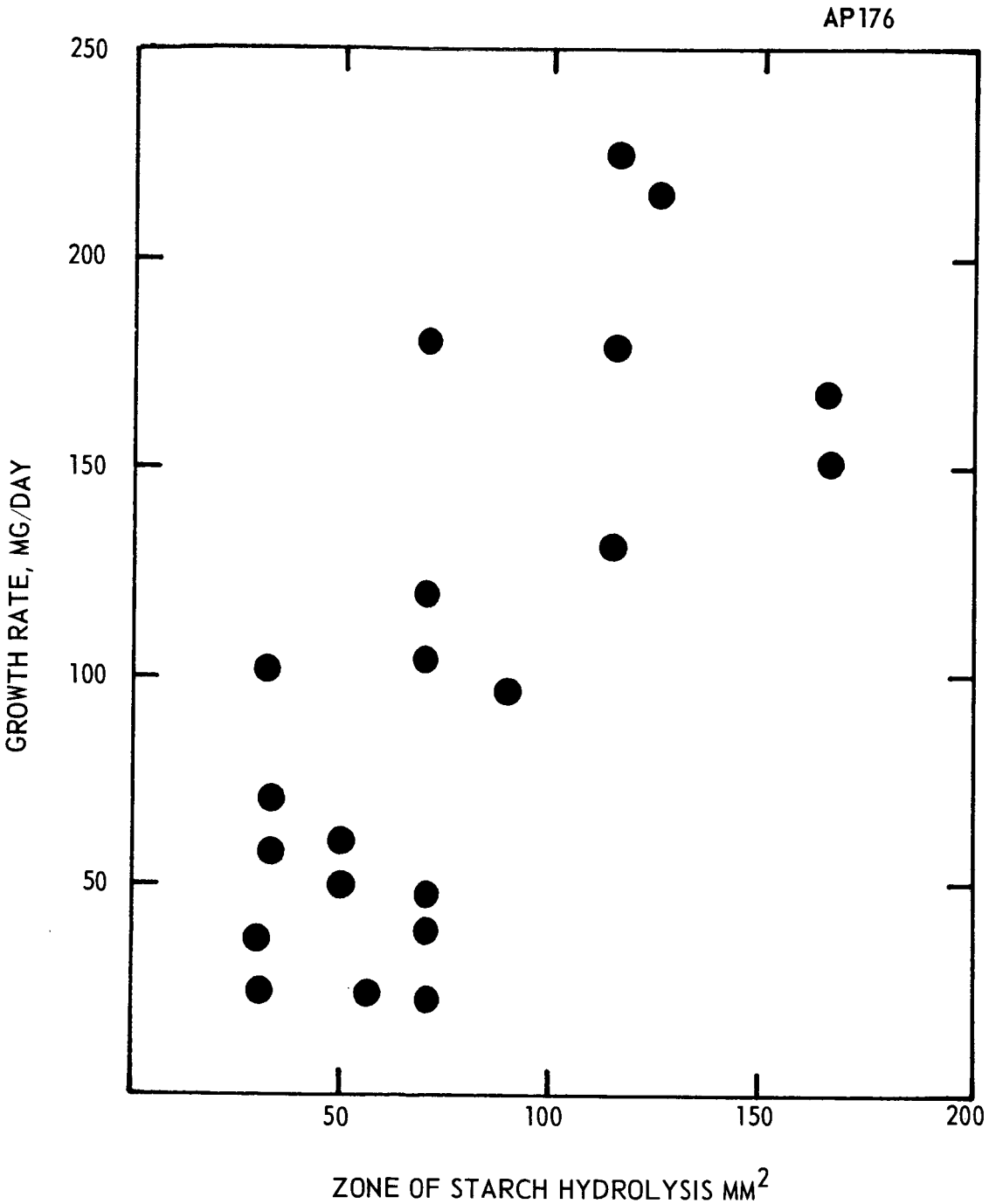


FIGURE 3 THE RELATIONSHIP BETWEEN THE GROWTH-RESTORING AND AMYLASE RESTORING ACTIVITIES OF NUCLEOTIDES AND RELATED COMPOUNDS.

III. NON-AQUEOUS AND MODIFIED AQUEOUS MEDIA

A. The Extent of *Penicillium* Spore Development in Organic Solvent-Water Media: 7 Months Data

In November, 1966, it was reported that small yields of germinating *Penicillium* spores were observed in various solvent-water systems after 30 days incubation with standard glucose-peptone-yeast media at 25°C. This study was paralleled with a demonstration that the enzymes peroxidase and catalase retained marked activity in such solvent media. Now, after an additional period of about 7 months, the same cultures have been reexamined. From the original 22 showing spore activity out of 46 media set up, only 7 remain active (Tables 5 to 8) and 9 additional appear to have died. Initially, alcoholic media were more supportive; 10 out of 12 containing germ tubes. At 200 days, only one has retained activity. In acidic media, however, 6 were active initially, and 4 remain so.

The low yield of germination (1% was highest recorded) suggests that the process could have been supported by traces of water present in the most concentrated solvents. If this is true, the ability to extract and use the small quantity of water available in some of this media is a remarkable capability. Solvolytic processes not involving free water might be assisted to a limited extent by solvolytic reactions not requiring water, or by mixed solvolysis. Further exploration of this area will involve a water/solvent proportion range.

Table 5. Germination of Penicillium Conidiospores

After 200 Days in Acid Nutrient Media*

System (% Composition)	Growth Activity	
	30 Days	200 Days
Formic Acid		
98	+	-
90	+	-
50	0	-
Acetic Acid		
98	+	+
90	+	+
50	+	0
Propionic Acid		
99+	0	0
90	0	0
50	0	+
Formamide		
99	0	0
90	0	0
50	+	+

* 5 g/l glucose, 1 g/l each peptone and yeast extract.

+ = continuing germination

0 = none

- = death

Table 6. Germination of Penicillium Conidiospores After 200 Days in Alcoholic Nutrient Media*

System (% Composition)	Growth Activity	
	30 Days	200 Days
Methanol		
100	+	-
90	+	-
50	+	+
Ethanol		
98	0	0
90	0	0
50	+	0
Ethylene Glycol		
96	+	0
90	+	0
50	+	-
Glycerol		
98	+	0
90	+	0
50	+	0

* 5 g/l glucose, 1 g/l each peptone and yeast extract.

\+ = continuing germination

0 = none

\- = death

Table 7. Germination of Penicillium Conidiospores After 200 Days in Non-Aqueous Binary Nutrient Media *

System (% Composition)		Growth Activity	
Solvent A	Solvent B	30 Days	200 Days
Methanol (90)	Formic Acid (10)	0	-
Methanol (50)	Formic Acid (50)	0	-
Methanol (90)	Formamide (10)	+	+
Methanol (50)	Formamide (50)	+	+
Dioxane (50)	Formic Acid (50)	0	-
Dioxane (50)	Formamide (50)	+	0
Dioxane (50)	Methanol (50)	0	0

* 5 g/l glucose, 1 g/l peptone and yeast extract.

+ = continuing germination

0 = none

- = death

Table 8. Germination of Penicillium Conidiospores After 30 Days in Two Phase Binary Solvent Nutrient Media*

System (1:1 by Vol.)		Growth Activity	
Solvent A	Solvent B	30 Days	200 Days
Hexane	Water	+	0
	Methanol	+	0
	Formic Acid	0	-
	Formamide	0	-
Benzene	Water	+	0
	Methanol	0	0
	Formic Acid	0	-
	Formamide	-	-

* 5 g/l glucose, 1 g/l peptone and yeast extract

+ = continuing germination

0 = none

- = death

B. Vital Activity of Penicillium Conidiospores in Liquid Ammonia at 233°K After Six Months

In the last report, morphological evidence (Figure 4) was presented for germination of Penicillium conidia at 233°K in a nutrient vehicle consisting of equal volumes of liquid NH_3 and 97% glycerol which contained yeast extract, peptone, glucose and ATP (10, 10, 50 and 1 g/l, respectively). Previous observations have been successfully repeated, and are now reinforced by tracer evidence indicating incorporation of nucleic and protein constituents into the spore mass. Conidia of the resistant strain were divided into four groups and given the following treatments:

a. none

b. 125°C for 24 hrs (heat-injured)

c. 5×10^6 rads Co^{60} γ-radiation (radiation-injured)

d. b + c (heat + radiation-injured)

From each group treated, 3 sets of 75 mg inocula were introduced into 21 ml of culture medium containing 100μC thymidine-H^3 (15C/mmole), 100μC uridine- 5,6-H^3 (20C/mmole) or 50μC each of L-phenylalanine-2,3-H^3 (2.5C/mmole) and L-leucine-H^3 (1.8C/mmole). After 6 months at 233°K, the ammonia was allowed to boil off, the medium was decanted, and the entire spore mass was extracted with 1 M trichloroacetic acid followed by a saline wash. The spores were then suspended in methanol-toluene and counted with liquiflor in a Nuclear--Chicago "Unilux" Scintillation Counter. Background was about 25-40 counts/min (CPM). Although the data show variable effects of injury upon incorporation, it is clear that incorporation processes have proceeded most in untreated spores (Table 9).

With morphological and biochemical evidence combined, there can be little doubt that liquid ammonia environments can sustain to some degree the vital activities of a terrestrial organism. These data together with previous observations on growth of organisms in ammonia-water at ordinary temperatures support a serious consideration of Jupiter as an abode of recognizable life.

C. Preliminary Experiments with Heme Enzymes

To block out areas of interest for the study of biochemical behavior under extreme environments, we initiated a survey of solvent media using peroxidase and catalase at test enzymes. In the last report, activity was demonstrated with peroxidase in various alcohol-water and carboxylic acid-water solvents, and even in some nearly anhydrous and aprotic solvents.

The reaction mixture, as before, contained 10 ml of solvent, 0.2μ moles of peroxidase, 0.5 mmoles of substrate, 0.5 mmoles of H_2O_2, and 2 mmoles H_2O. Turbidity prevented direct photometric determinations of reaction course; hence, until techniques have been devised for reliable optical methodology, a qualitative picture must suffice (Table 10). Guaiacol, benzidine, 1,8-diaminonaphthalene, and 3,4-dimethylphenol are all converted readily to colored oxidation products when, and only when, both peroxidase and H_2O_2 are supplied in water, 15M NH_4OH, and 10M N,N-diethylaminoethanol; this does not occur in hydrazine. A few observations also suggest novel interactions in 15M NH_4OH of peroxidase with metals. Elementary cobalt in 15M NH_4OH with H_2O_2 yields highly colored solutions more rapidly when peroxidase is present. On the other hand,

the formation of MnO_2 from elementary Mn is stopped by the presence of peroxidase. Metallic Hg is converted into black HgO by peroxidase, whereas none appears with H_2O_2 only.

Preliminary tests for catalase activity were based solely upon visual observation of O_2 bubble evolution when, to 10 ml of solvent, 0.5 mmoles of H_2O_2 and 0.04 μ moles of catalase were added. At room temperature, ca 295°K, a variety of solvents supported activity (Table 11), but even more interesting, activity was readily observable in a number of solvents at 233°K (Table 12). At 195°K, no activity was observed after 45 min., however longer times and instrumental methods may be required for detection of O_2 evolution. It is clear however that catalase, like peroxidase, can retain its function under conditions highly alien to the cell.

Table 9. Trichloroacetic Acid - Insoluble Residue from Spores Incubated in Liquid NH_3 (233°K) with Tritiated Metabolites

Label	Treatment of Spores: Heated 125°C/24 hr	$Co^{60}\gamma$ 5 x 10^6 rads	H + γ	None
	β activity --- counts/min/75mg			
Thymidine-H^3	5,258	742	600	10,980
Uridine-5,6-H^3	3,750	1,475	1,250	12,433
L-Phenylalanine-2,3-H^3 + L-Leucine-H^3	1,800	3,241	2,141	10,300

Table 10. Oxidation of Selected Substrates By Peroxidase

Substrate	Color of Oxidation Product in H_2O	15M Ammonia	10M Hydrazine	10M Diethylaminoethanol
Guaicol	+(Red Brn.)	+(Brown)	-	+(Red)
Benzidine	+(Blue gray)	+(Brown)	-	+(Brown)
1,8-Diaminonapthalene	+(Purple)	+(Red purple)	-	+(Purple)
3.4-Dimethylphenol	+(Orange)	+(Red orange)	-	+(Orange)

Table 11. Activity of Catalase at Room Temperature in Experimental Solvent Systems.

Solvent	Activity (presence of O_2 bubbles after 5 min)
H_2O	++
Glacial formic acid	-
Formic acid (90%) + H_2O	
Formic acid (50%) + H_2O	++
Methanol	-
Methanol (90%) and H_2O	++
Methanol (50%) and H_2O	++
Acetic acid (90%) + H_2O	+
Acetic acid (50%) + H_2O	++
Propionic acid (90%) + H_2O	+
Propionic acid (50%) + H_2O	+
Ethanol	++
Ethanol (90%) and H_2O	++
Ethanol (50%) and H_2O	++
Ethylene glycol	-
Ethylene glycol (90%) + H_2O	+
Ethylene glycol (50%) + H_2O	++
Glycerol	++
Glycerol(90%) + H_2O	++
Glycerol (50%) + H_2O	++
Formamide	+
Formamide (90%) + H_2O	+
Formamide (50%) + H_2O	+
Methanol (90%) + Formic acid	-
Methanol (50%) + Formic acid	+
Methanol (90%) + Formamide	+
Methanol (50%) + Formamide	+

Table 12. Activity of Catalase at 233°K in Experimental Solvent Systems

Solvent	Activity presence of O_2 bubbles after: 15 min.	45 min.
Formic Acid	-	-
Formic A (90%) + H_2O	-	-
Formic A (50%) + H_2O	±	-
Methanol	-	-
Methanol (90%) + H_2O	++	++
Methanol (50%) + H_2O	-	-
Acetic Acid (90%) + H_2O	-	-
Acetic Acid (50%) + H_2O	-	-
Propionic acid (90%) + H_2O	-	-
Propionic acid (50%) + H_2O	-	-
Ethanol	++	++
Ethanol (90%) + H_2O	++	++
Ethanol (50%) + H_2O	-	-
Ethylene glycol	-	-
Ethylene glycol (90%) + H_2O	±	-
Ethylene glycol (50%) + H_2O	-	++
Glycerol	-	++
Glycerol (90%) + H_2O	-	++
Glycerol (50%) + H_2O	-	+
Formamide	-	-
Formamide (90%) + H_2O	+	+
Formamide (50%) + H_2O	-	-
Methanol (90%) + Formic Acid	-	-
Methanol (50%) + Formic Acid	-	-
Methanol (90%) + Formamide	-	-
Methanol (50%) + Formamide	-	-

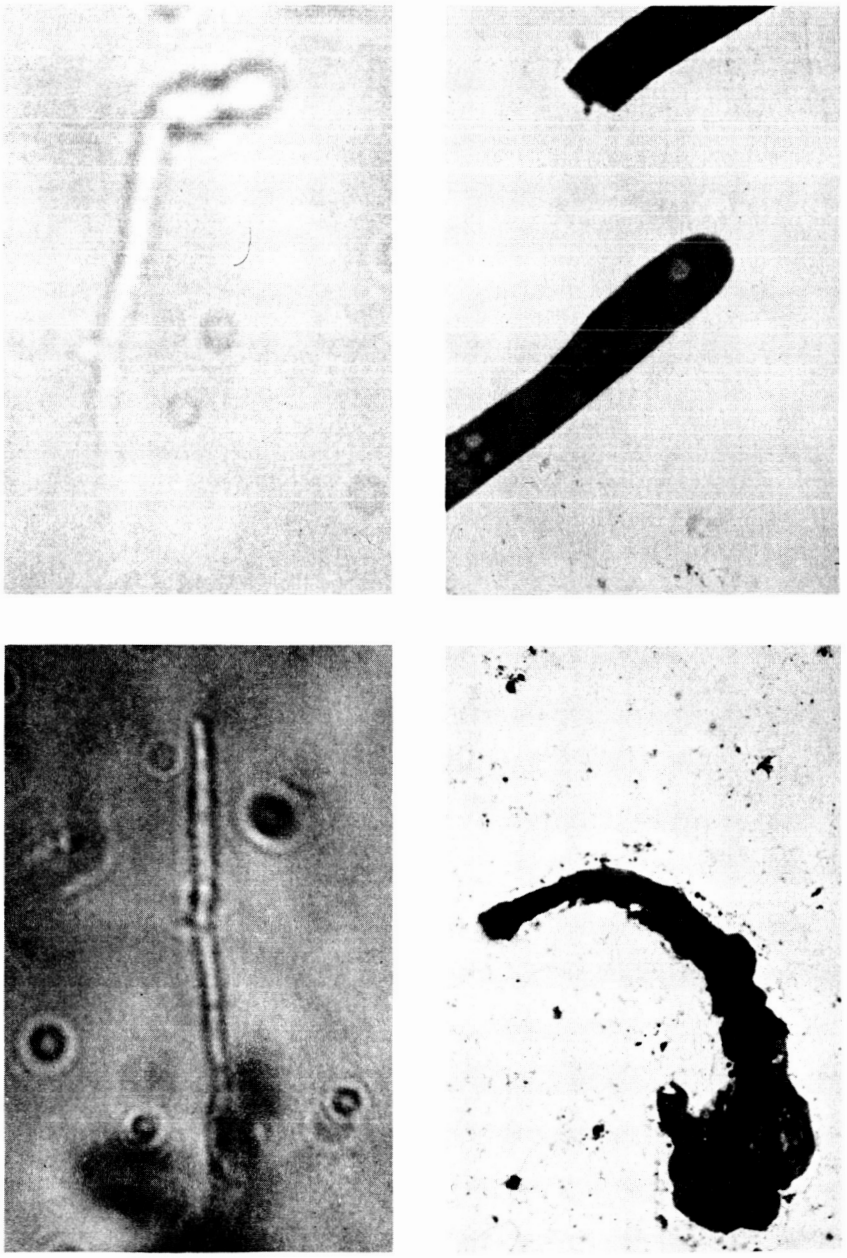


Figure 4 Morphological demonstration of Penicillium spore germination in liquid NH_3 media at 233°K.

Upper Left: Entire germinating spore showing germ tube and branch (950X).

Upper Right: Hyphal tip showing vacuole–like bodies of low electron opacity (10,000X E.M.).

Lower Left: Hyphal tip (950X light).

Lower Right: Encrusted germinating spore (5000X E.M.).

IV. TOXICANTS

A. Metal Tolerance and Accumulation in Penicillium: General Comparisons of Wild Type and Tolerant Strains

The last report disclosed the successful growth of mutant Penicillium on nutrient media slurried with lead, boron or other metallic or non-metallic elements. At present, this number of elements has been increased by an additional 11 to a current total of 30 (Figure 5).

A number of these elements are generally regarded to be cellular poisons--Hg, Cu, Cd, Pb, Co, for example,--and it might be assumed that tolerance to them is a matter of exclusion. That this is incorrect is shown by the lack of graphical correlation between inhibition of growth in the presence of the element and its content in the tissue (Figure 6).

The figures given are based upon 30-day cultures. It should be noted that although growth nearly ceases after about 10 days, metal accumulation continues for at least 2-3 weeks more. The analytical picture as illustrated by Cu, Cd, Zn and Ni in younger vs. older tissues is:

	10 Days	30 Days
	% Dry wt	
Cu	4.3	23
Cd	0.5	5.4
Zn	1.7	21
Ni	7.2	13

At 30 days, the Cu-mycelium is deep blue, even when dry. The location of Cu in the cells was readily established by flooding washed mycelium with $(NH_4)_2S$ solutions under the microscope, thereby converting the metal to the sulfide in situ (Figure 7).

The distinctive character of our tolerant mutant strain is further illustrated by a comparison of its growth with wild type P. notatum in the GPY-element media (Table 13). Although both strains show comparable amounts of growth in metal-free GPY-medium culture the wild type grows less in every instance when free elements are present.

Table 13. Relative Growth of Wild Type and Salt-Tolerant Strains on Metal Media*

	Strain	Growth mg/2 wks	W/T **	Sporulation
Control	W	499 ± 8	1.10	+
	T	452 ± 26		+
Al	W	287 ± 10	0.67	+
	T	431 ± 11		+
B	W	201 ± 10	0.45	+
	T	448 ± 10		+
Bi	W	313 ± 12	0.78	+
	T	401 ± 9		+
Cd	W	200 ± 28	0.51	+
	T	389 ± 30		+
Co	W	0	0	0
	T	258 ± 10		+
Cr	W	227 ± 5	0.51	+
	T	444 ± 6		+
Cu	W	18 ± 4	0.04	0
	T	485 ± 10		+
Fe	W	61 ± 5	0.32	+
	T	188 ± 12		+
Mo	W	36 ± 20	0.34	+
	T	105 ± 14		+
Nb	W	256 ± 5	0.80	+
	T	322 ± 15		+
Ni	W	0	0	0
	T	103 ± 30		+
Si	W	243 ± 35	0.58	+
	T	420 ± 19		+
Sn	W	275 ± 11	0.62	+
	T	442 ± 16		+
Ta	W	216 ± 15	0.62	+
	T	349 ± 5		+
Zn	W	123 ± 5	0.27	0
	T	461 ± 5		+

* Nutrient broth (5g/l glucose, 1g/l peptone, 1g/l yeast extract) added to metal powders.

** Ratio of mycelial mat weight of wild strain to salt-tolerant strain.

Elements used experimentally in culture media for successful growth of Penicillium form spores are shown in bold face type.

H																He
Li	Be											B	C	N	O	F	Ne
Na	**Mg**											**Al**	**Si**	P	S	Cl	Ar
K	Ca	Sc	**Ti**	**V**	**Cr**	**Mn**	**Fe**	**Co**	**Ni**	**Cu**	**Zn**	Ga	**Ge**	As	Se	Br	Kr
Rb	Sr	**Y**	**Zr**	**Nb**	**Mo**	Tc	**Ru**	**Rh**	Pd	**Ag**	**Cd**	**In**	**Sn**	Sb	Te	**I**	Xe
Cs	Ba	La	Hf	**Ta**	**W**	Re	Os	Ir	Pt	**Au**	**Hg**	Tl	**Pb**	**Bi**	Po	At	Rn
Fr	Ra	Ac															

FIGURE 5 PERIODIC TABLE OF THE ELEMENTS

AP 188

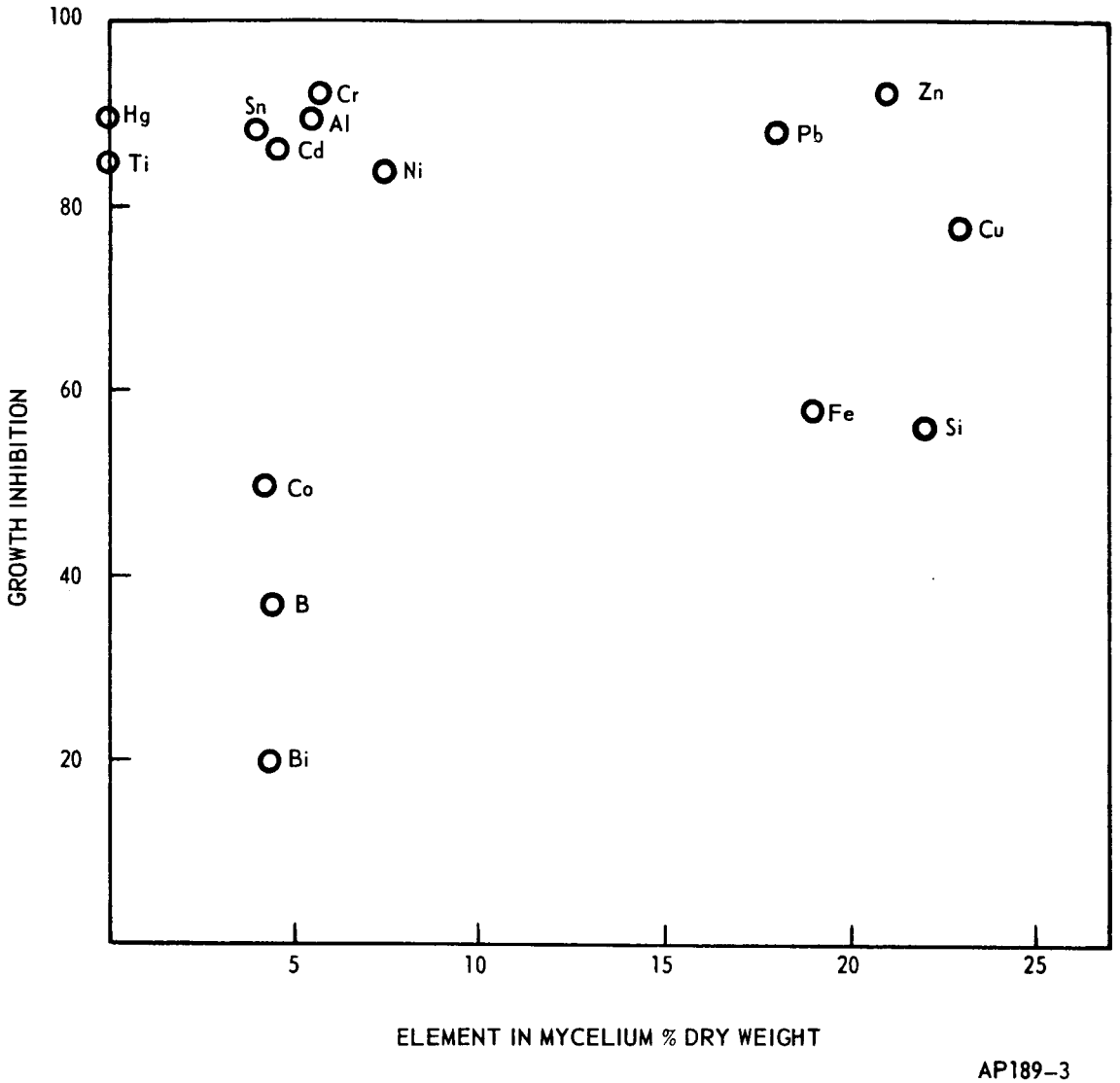


FIGURE 6 DEMONSTRATION OF THE NON-CORRELATION OF GROWTH INHIBITION IN METAL MEDIA WITH THE AMOUNT OF SPECIFIED METAL TAKEN UP.

Figure 7 Cytochemical demonstration of copper uptake in Penicillium cells (950X). Cu fixation by $(NH_4)_2S$. Note variation in Cu from cell to cell. Tissue averaged 23% Cu by weight and was blue.

B. Growth of Salt-Tolerant Penicillium notatum in Boron-Rich Media

Boron toxicity in vascular plants is well documented as a phenomenon, although specific injury mechanisms have not yet been established. Severe inhibition of many Angiosperms can be brought about by an ambient boron (borate) concentration of 25 mg/l or less. In some instances 5 mg/l or less suppresses growth severely. In Angiosperm tissue, levels of nearly 4000 mg/kg have been reported in leaves from plants grown in media containing 25 mg/l borate or less, although levels of appreciably less than 1000 mg/kg are more common.

Studies on the effects of toxic amounts of boron on lower plants, such as fungi, are scarce. The literature does give evidence that different species of fungi vary in their requirements for and their tolerance to boron. Foster observed that his Aspergillis niger No. 67 was sensitive to 400 mg/l boron while another strain of A. niger required 2000 mg/l boron for comparable inhibition. Bowen and Gauch, in their study on the nature of boron toxicity, found that 50 mg/l borate inhibited the growth of Saccharomyces cerevisiae; that 1300 mg/l inhibited their strain of A. niger; and that 4000 mg/l borate was required to produce a significantly toxic effect in Penicillium chrysogenum. Luchetti reported the growth of a Cephalosporium, isolated from borax-rich soils, in culture media containing 10% sodium borate and 12.5% boric acid.

Investigations reporting upon boron tolerance in microorganisms have not been accompanied by analyses of boron content, except for one instance. Bowen and Gauch found that Saccharomyces incubated with 300 mg/l borate reached near saturation after about 2 hours, assaying 900 mg/kg dry weight. They also found 1886 mg/kg of boron in cells of P. chrysogenum cultured in 4000 mg/l of borate.

Continuing the study of the growth of fungi in extreme chemical environments, it was found that a strain of Penicillium notatum, isolated from a saturated calcium acetate nutrient medium, had been inadvertently selected for an ability to grow in nutrient media saturated with KCl and other salts. Extension of this observation showed this Penicillium to have an unusual tolerance to other harsh conditions including low pH (2-4N H_3PO_4), and to various elementary metals and non-metals usually regarded as toxic.

This paper is concerned with the tolerance of salt-adapted Penicillium toward boron in its several oxidation states.

Conidiospores of wild type Penicillium notatum and of a resistant strain isolated in this laboratory were used. The selection of the resistant strain, its culture on glucose-peptone-yeast medium, and some aspects of its salt and nutritional relations have been described. In the present experiments, 30 mg of conidiospore inocula were cultured at 23°C in a medium containing 5 g/l glucose, 1 g/l Bacto-peptone and 1 g/l yeast extract. Replicate 100 ml volumes were used routinely. Into 100 ml volumes of nutrient medium, a boron source was introduced; this was either 15 g of powdered high purity boron, 5 g of crystalline orthoboric acid, or 0.8-1.6 g of sodium borohydride. In each case the pH was adjusted to 6.0. Since sodium borohydride hydrolyzes slowly at near neutral pH, it was decided to distribute borohydride in the medium more or less uniformly over the incubation period; it was not added all at once, as were elemental boron and boric acid, but was added in uniform portions on alternate days to give the final concentrations specified.

Growth was recorded as time for appearance of visible mycelium and mycelial dry weight after 14 days. Boron content was determined by emission spectrography. Mycelium grown on elementary boron-nutrient medium was washed for 30 minutes in running distilled water, digested with 3% HCl for 1 hour, cooled and filtered to remove any contaminating boron particles, and the filtrates analyzed. Mycelium grown in boric acid media was washed with 0.01N NaOH for 30 seconds, with cold distilled water for 30 minutes, and the residue analyzed. Mycelium from borohydride cultures required only the 30 minute wash in distilled water.

One experiment was set up to test for volatile boron compounds. Sterile polyethylene bottles were set up with or without nutrient, boron and spores. Over the culture liquid, 50 ml in volume, at a height of about 5 cm, a 2 x 1 cm rectangle of Whatman No. 1 filter paper that had been soaked in 0.01N NaOH was suspended. Cultures were sealed for 7 days, opened and the papers tested for H_3BO_3. Feigl's curcumin test was carried out after application to each paper of 0.1 ml glacial acetic acid.

For comparative purposes, cultures were set up with boron, aluminum and other high purity elements in 50 ml nutrient volumes but incubated for 30 days before weighings and analyses were carried out.

Wild type and mutant Penicillium grew at comparable rates in standard glucose-peptone-yeast extract (GPY) and produced typical green areas of sporulation (Table 14). Macroscopic colonies were evident within two days. In contrast, a lag or induction period of 12 days was necessary for perceivable growth of wild type in a GPY-Boron medium. During the next two days, growth proceeded at one-quarter control rate. The tolerant

strain was delayed only slightly and then grew at one-half of control rate. Sporulation was limited to the tolerant strain, but commonly the conidia and spores that it produced were violet instead of green. Individual hyphae also showed violet coloration. When extracted in 95% ethanol-3% HCl, the pigment absorbed at λ 260 and 540 mμ. The pigment was decolorized in alkaline medium (pH>8) or by ascorbic acid in acid solution. Efforts to isolate this substance are being made.

In GPY-Boric Acid the wild type failed completely but the tolerant strain grew comparatively well, forming a dense pure white mycelium without spores. In GPY-$NaBH_4$, however, the tolerant strain sporulated readily and, in older cultures, became tinged with violet.

In GPY-Boron medium, the tolerant Penicillium accumulated 1600 mg B/100 g dry mycelium in two weeks. This corresponds to 1.6% of dry matter, or 16,000 ppm. On GPY alone, no B was detected spectrographically, limiting the maximum content to available analytical limits, ca 10 mg/100g, or 100 ppm (Table 15). GPY-Boric Acid medium supported poorer growth and yielded mycelium containing 2000 mg B/100 g dry wt. When sodium borohydride, $NaBH_4$, was supplied, growth was maintained at a comparatively high level although B in this reduced form amounted to 2-4 fold more than was in solution as H_3BO_3. After two weeks, up to 2300 mg/100 g dry wt of B accumulated in the mycelium. In H_3BO_3 and $NaBH_4$ media, approximately 0.5-1% of the total "available" B was incorporated into the mycelium. "Available" B was determined as the B in solution in saturated H_3BO_3 and as the B present as $NaBH_4$ in solutions of specified concentration. The solubility of elementary B in water at pH 6-7 is too low to permit comparable calculations of available B or percentage incorporated.

The refractory character of elementary B raised questions about the mode of accumulation. Two week old GPY-Boron medium gives the positive curcumin test of Feigl, showing that H_3BO_3 has been formed but results with borohydride indicated that the organism could also accumulate boron from a reduced source. Examination of Table 15 shows that mycelium grown in media containing 1.6 g/100 ml of $NaBH_4$ contains somewhat more boron than was found in the mycelium grown in the presence of 5 g/100 ml of H_3BO_3. It therefore seems clear that the efficiency with which boron is taken up differs in the two oxidation states and that borohydride is taken up without total hydrolysis to boric acid. This contrast is even more pointed when it is recalled that a concentration of 1.6 g $NaBH_4$/100 ml was the integrated concentration over the incubation period and was far in excess of the day to day level.

Although there could be no question about the spontaneous formation of boric acid from elementary boron, the established reducing activities of the genus Penicillium toward As, Se, and other members of Groups V and VI leave open as an alternative possibility the solubilization of elementary boron by means of biological reduction. Although our test for the presence of volatile boron compounds could not be used to characterize their oxidation-reduction state, it showed clearly that such substances do not arise spontaneously in sterile, uninoculated boron-rich nutrient broth (Table 16).

In view of the remarkable degree of boron accumulation, it was of interest to see whether other elementary substances, including the related metal, aluminum, behaved similarly. Experimental conditions were somewhat different in the comparative study than in standard experiments described thus far, but all relevant comparisons--GPY medium alone and

GPY-element--were run simultaneously (Table 17) at a pH of 6-7. In GPY-Boron, growth in 30 days was reduced by about 40%. In previous experiments, the growth rate in GPY-Boron was reduced by 50% (Table 14), and total growth 54% (Table 15) on the basis of 14 day incubation periods. The absolute amount of growth was limited by the smaller volume of medium. Over aluminum and zinc, growth was less than one-tenth control. Cadmium and titanium were similar to one another yielding somewhat more than 10% of control growth. GPY-silicon allowed about one-half of control growth. The greatest uptake was found in the presence of aluminum, boron and silicon respectively. Mycelium grown with zinc, cadmium and titanium showed comparatively poor growth and low metal uptake.

The present data point strongly to a genetic determinant in boron tolerance but one that is more generally related to conditions favoring high performance under stress. If there is a more direct relationship between the ability to grow in ultrasaline media, as represented by saturated calcium acetate, and the ability to grow in boron-rich media, it is at present obscure.

Furthermore, the ability to grow in strongly acidic media (phosphoric acid as noted above) or on metallic elements such as zinc and cadmium all support the concept of a generalized stress selection mechanism.

Table 14. Growth of Salt-Tolerant Penicillium

In Boron-Rich Media.

Wild type or resistant conidiospores were incubated at 23°C at pH 6.0 in glucose-peptone-yeast extract (5,1 and 1 g/l respectively) medium alone or containing 15 g/100 ml of boron or 5 g/100 ml boric acid.

Dry weights were taken after a two-week incubation period. "Induction Period" refers to time for visible growth. "Growth Rate" refers to fresh wt increase from the end of the induction period to day 14.

Boron provided in medium	Conidiospore stock	Growth Responses		
		Induction period (days)	Growth rate fresh wt/day after 14 day incubation	Sporulation after 14 days incubation
None (control)	Wild type	1	129 ± 11	+
	Tolerant	1	122 ± 13	+
B (15 g/100 ml)	Wild type	12	29 ± 4	-
	Tolerant	2	31 ± 7	+
H_3BO_3 (5 g/100 ml)	Wild type	>16	0	-
	Tolerant	5	48 ± 5	-

Table 15. Boron Content of Two-week Old Penicillium Mycelium Incubated in Boron-Rich Media.

Conidiospores of tolerant strain were grown on standard glucose-peptone-yeast extract medium at 23°C at pH 6.0 for two weeks, the mycelium recovered and B determined spectrographically.

Boron provided in medium	Boron in 100 ml of medium as B	mg mycelium produced in two weeks		B content of dry mycelium	Total Boron in mycelium grown (as B)
	mg	fr wt	dry wt	mg/100gm	mg
none	0	1800	181	<10	<0.1
B (15 g/100 ml)	Saturated <<1	796	99	1600	1.6
H_3BO_3 (5 g/100 ml)	Saturated ca 100	396	49	2000	1.0
$NaBH_4$ (0.8 g/100 ml)	23.1	1209	201	500	1.0
$NaBH_4$ (1.0 g/100 ml)	28.9	1174	196	800	1.6
$NaBH_4$ (1.6 g/100 ml)	46.2	1079	179	2300	4.1

Table 16. Detection of Volatile B-Compounds Formed by *Penicillium* in Nutrient plus Elementary B

Each vessel contained a 2 x 1 cm rectangle of filter paper soaked in 0.01N NaOH suspended 5 cm over the liquid medium. After one week papers were collected, acidified and tested for borate with curcumin.

Polyethylene vessel	Medium	Boron powder	Spore inoculum	Curcumin test
1	sterile water	-	-	-
2	sterile nutrient	-	-	-
3	sterile nutrient	+	-	-
4	sterile nutrient	-	+	-
5	sterile nutrient	+	+	+

Table 17. A Comparison of Growth and Uptake by Penicillium in Thirty Day Cultures over Selected Elements

Conidiospores of the tolerant strain were incubated in 50 ml of glucose-peptone-yeast extract at a pH of 6-7 for thirty days, and the mycelium subsequently recovered, washed, dried and analyzed spectrographically.

Element (5 g/ 50 ml)	Fresh wt in 30 days in 50 ml	Element content in dry mycelium after 30 days
	mg	mg/100 g
none	806	-
B	503	4300
Al	67	5400
Si	342	3500
Zn	63	1000
Cd	112	500
Ti	108	40

C. Experimental Paleobiology: New Observations on a Living Organism Structurally Related to Kakabekia umbellata Barghoorn, a Precambrian Microfossil

During an investigation of biological behavior in exotic and harsh environments, the ammonia-tolerant microflora in soils of different origins was studied. Ammonia tolerance is remarkably widespread, but even more remarkable was the appearance, in a soil specimen from Wales, of a small microorganism exceptional for its complex optical structure. This form did not obviously fall into ordinary categories such as "bacteria," "algae," etc. and its affinities remained totally obscure until Kakabekia umbellata Barghoorn (a Precambrian microfossil), was described by Barghoorn and Tyler. This fossil was found in a chert deposit ca 2×10^9 years of age in the Gunflint range of Southern Ontario. Deposition at this site was apparently associated with a change, in the microhabitat at least, from reducing to oxidizing conditions. Barghoorn and Tyler noted that the affinities of K. umbellata could not be readily assigned "...to a living counterpart, provided any exists." When petrographic sections containing Kakabekia were compared "side-by-side" with the living microorganism cultured under ammonia from the Welsh soil, there was no doubt that they were similar in size and optical detail. Within the respective morphological ranges of fossil and living populations, there were individuals that were essentially identical. At their points of closest correspondence, the following description fits both forms: a umbellate form 5-10 μ in diameter possessing a centrally attached stalk ca 5-15 μ in length with a terminal swelling or enlargement. The umbrella or crown is more or less polygonal, heavily rimmed, and five to eight-parted. The partitions radiate from the center of the crown and suggest the ribs of an umbrella or spokes of a wheel. The stalk is often straight

but sometimes appears twisted or "hawser-like" (Figure 8). Variations in both populations exist, and the living forms (Figure 9) often possess a circular crown and are sometimes seen without a stalk. Nevertheless, the similarities have warranted the designation "Kakabekia-like" for the living form. Further credibility was lent to the idea of a relationship between Kakabekia umbellata Barghoorn and our similar contemporary form by the presence of alga-like bodies morphologically identical to modern blue-greens (such as the Oscillatoriaceae) both as microfossils in Gunflint chert and as part of the living microflora of the Harlech soil samples. The observation of normal-appearing microflora in our cultures also minimizes the likelihood that the Kakabekia-like forms are merely the result of the action of ammonia upon common soil organisms. Further studies on this unusual organism have now been carried out.

Soil Samples. The locations which have been sampled and examined for Kakabekia-like forms include loam soils from Western Europe, the Dakotas, New York, and Pennsylvania; peats from Northern England; sands from Florida; and laterites from the Guianas, Curaçao, and other Caribbean locations. Sampling involved only the upper 1-3 cm of the A horizon. In no case was there any indication of a Kakabekia-like form when these soils were cultured 30 days at 25°C on a 2 percent agar containing 1.0 percent Bacto-peptone plus 0.5 percent beef extract under an atmosphere consisting of 50 percent NH_3 in air. Two samples from Harlech, Wales, yielded Kakabekia-like specimens under the foregoing and similar cultural conditions (Table 18). The first (H-1) was collected in August, 1964, at the inside base of the wall of Harlech Castle. The second (H-2), collected in August, 1965, was taken from the center of the castle courtyard. After six to seven months' storage in air-dry conditions, H-1 yielded far more umbellate forms

upon cultivation than H-2. H-1, however, fell markedly in yield during storage. For comparison, samples of local sandy loam soil from Tarrytown, New York (T-1) was devoid of Kakabekia-like organisms.

Although the physical and chemical characterization of soils yielding Kakabekia-like forms has not been completed, their major chemical features have been determined (Table 19). The local soil sample (T-1) agrees well in composition with various loams and has been used therefore as a reference sample. On this basis, H-1 and H-2 are grossly alike in containing more C, H, N, and Al and less K_2O, TiO_2, Na_2O, and Rb than T-1. All three samples contain similar amounts of Cr. Many elements that were lower in H-1 than T-1, Fe, Ca, Cu, for example, were higher in H-2 than in T-1. Conceivably the differences shared by H-1 and H-2 relative to T-1 may have qualitative significance and those between H-1 and H-2 quantitative significance with respect to yield of Kakabekia-like forms.

Soil specimens H-1 and H-2 both contain objects within the size range of the Kakabekia-like forms which fact might serve as a source of confusion (Figure 10). Included are structures resembling tetraspores of unicellular algae or sporangia of ascogenous yeasts (upper row, left); smooth, polished granules of quartz which may have a striking degree of radial or concentric structural detail as a result of cracking or differential solution (upper row, right); and crystals that form only in the presence of ammonia (lower row). Confusion is heightened by the tendency of highly pointed umbellate crowns of Kakabekia-like forms to become folded (Figure 10).

Culture. The cultivation of Kakabekia-like forms from soil samples H-1 and H-2 has been the object of concerted efforts. Inorganic nutrients, amino acids and vitamins separately, Eagle-Hanks medium, complex

organic fluids (including blood, milk, coconut milk, and urine) have all been tested. Liquid culture media, shaken and stagnant, and semisolid media have been compared. Differential sterilization of sample H-1 was also attempted (Table 20). Like the NH_3-tolerant green algae and diatoms present, Kakabekia-like forms were more heat- and radiation-sensitive than blue-green algae or bacteria.

In broth culture, the time required for appearance of Kakabekia-like forms from sample H-2 was a sensitive indication of nutritional balance (Table 21). Without ammonia (i.e. in nutrient broth, aerobic culture) no forms had appeared after 75 days. In NH_4OH, no forms were observed in the absence of peptone, even when silicate and glucose were provided. Responses in 5 and 10 M NH_4OH to peptone, silicate and glucose were similar: at the low peptone level, 1 gm/l, organisms were observed either with 1 gm/l of silicate plus glucose or with 10 gm/l of silicate even without glucose, but they appeared only after many weeks of incubation. At the high level of peptone, Kakabekia-like forms appear rapidly when glucose is present, irrespective of silicate supply, but appear only slowly when provided silicate at 10 gm/l, in the absence of glucose. Possibly the low vigor of this sample and the requirement for a complex N-source, peptone, are related.

The more vigorous inoculum H-1 provides Kakabekia-like forms rapidly in glucose-ammonia-peptone broth, and even shows activity when provided only with glucose and ammonia (Figure 11). Note that the increase in number is linear, attains a maximum and then declines. These features will be considered (below) in relation to ontogeny.

More complex media containing blood, urine, coconut milk or milk were at best no improvement over glucose-ammonia-peptone, and, in some cases, were highly inhibitory.

Ontogeny. Kakabekia-like forms are not in evidence in dry soil samples, nor can they be seen in typical morphology in less than about ten days under the best culture conditions thus far devised. It is obvious that the pre-umbellate stage of this organism is quite different in organization. Between the 5th and 10th day of culture on glucose-peptone agar a number of objects have been observed which might constitute a pre-umbellate developmental series (Figure 12). One of these (far right) is readily seen as an antecedent of the umbellate form; the others are arranged speculatively. All of these structures have in common an external wall or ring which remains more or less constant in thickness. This implies that morphogenesis proceeds by intercalation of materials into this ring, rather than by a "stretching" process. Previously, this ring was thought to be siliceous in umbellate specimens. No intermediate between the tripartite stage and the "mature" 6 to 8-parted forms have been observed. On the other hand these pre-umbellate forms are rarely seen in cultures relatively abundant in umbellate structures (the tripartite structure has never been found with the more mature stages). Upon prolonged cultivation, the count of intact umbellate forms begins to decline, eventually falling to zero. The mature form disintegrates after several weeks in culture. Specimens showing ring and spoke structures in the process of disintegration have been seen (Figure 13). Because of the absence of a logarithmic phase in growth curves and the failure, during many hours of visual observation, to note any signs of a fission process, it is concluded that the ontogeny of this organism is complex and involves a distinct reproductive phase. This phase may have gone unrecognized because of the organism's small size and a structure rendering it difficult

to distinguish from the larger motile bacteria. Even the earliest presumptive developmental stage (Figure 12) could not in itself be identified with the later tripartite form were it not for the suggestive intermediate structures. A possible ontogeny for these Kakabekia-like forms is:

Small (ca 1μ) heavy-walled ellipsoid form (cyst, zygospore, zoospore)

↓

5 to 10 X enlargement and radial segmentation (mitosis or meiosis?)

↓

Stalk (stipe) "primordium"

↓

Mature umbellate form with stalk and bulb ⟶ gametogenesis or sporogenesis

↓

Senescence and degeneration.

It has been noted that the material within the spokes or septa of the umbrella is Feulgen- and aceto-orcein-positive and the stalk is sometimes "cellular" in appearance. No organized nuclear structure has been observed, however. Conceivably, genetic material from the umbrella migrates into the stalk, aggregates in the basal bulb, and is released as a reproductive stage.

It should be noted that this ontogenetic picute differs from that proposed by Barghoorn and Tyler based upon arrangement of their fossil specimens. It is, essentially, the inverse of their sequence which begins with the basal bulb as the direct progenitor of the umbellate stage. Both ontogenetic sequences are subject at present to the same limitations on actual observation of developmental processes in pure culture or in the field. It can be hoped that the "living fossil" will eventually resolve this problem.

Table 18. Sources of Kakabekia-like Organisms

	Soil Sample			
	H-1		H-2	T-1
Date of Collection	8/64		8/65	8/64-8/66
Age of Sample when tested (months)	6	18	7	1-6
Maximum Density of Kakabekia-like forms on nutrient agar under 50% NH_3-50% Air (N/cm^2)	70-150	10-15	5-10	0
Maximum density of Kakabekia-like forms on nutrient broth containing NH_3 under air (N/cm^2)	--	ca 400	ca 30	0

Table 19. Effects of Composition on the Appearance of *Kakabekia*-Like Forms in Liquid Media Inoculated with Soil H-2

NH_4OH moles/l	Peptone gm/l	Na_4SiO_4 gm/l	Appearance of *Kakabekia*-Like Forms (Time in Days) Glucose (gm/l)	
			0	10
0	0	0		
		1		
		10		
	1	0		
		1	>75	>75
		10		
	5	0		
		1		
		10		
5	0	0	>75	>75
		1	>75	>75
		10	>75	>75
	1	0	>75	>75
		1	>75	45
		10	50	45
	5	0	>75	10
		1	>75	10
		10	45	10
10	0	0	>75	>75
		1	>75	>75
		10	>75	>75
	1	0	>75	>75
		1	>75	45
		10	45	45
	5	0	>75	10
		1	>75	10
		10	35	10

Table 20. Effects of Heat and γ-Radiation Treatments on the Yield of Kakabekia-Like Forms and Other Microorganisms

Treatment (Soil H-1)	Organisms Found				
	Kakabekia-Like N/cm²	Diatoms	Blue-Green Algae	Green Algae	Bacteria
None	8	+	+	+	+
None	11	+	+	+	+
125°C, 1 hr.	6	+	+	+	+
6 hr.	0	-	+	-	+
24 hr.	0	-	-	-	-
10^5 rads $Co^{60}\gamma$	0	-	-	-	+

Table 21. Chemical Composition of Soil Samples

Tested for Kakabekia-like Forms

Analysis (mg/gm dry)	Soil H-1 Harlech (castle wall)	H-2 Harlech (courtyard)	T-1 Tarrytown (orchard)
Organic[1]			
C	106.1	46.0	24.8
H	15.3	6.7	5.5
N	9.2	9.2	5.0
Principal Inorganic[2]			
SiO_2	766.	779.	797.
Fe_2O_3	11.4	71.5	42.9
Al_2O_3	5.7	3.8	3.8
K_2O	4.8	9.6	24.0
CaO	1.4	56.0	14.0
TiO_2	1.1	2.9	4.3
MnO	1.0	2.6	1.0
P_2O_5	0.9	1.8	1.8
MgO	0.7	6.8	6.8
Na_2O	0.4	1.3	7.8
Trace Inorganic[3]			
Ba	0.10	0.20	0.20
Rb	0.10	0.20	0.30
Cr	0.08	0.08	0.08
Cu	0.02	0.80	0.20
Ni	0.02	0.08	0.04
B	0.01	0.03	0.03
Zr	0.00	0.03	0.20
V	0.00	0.08	0.10
Pb	0.00	0.10	0.04

1. By combustion; 2. By spectrography, calculated as oxides;

3. By spectrography Sr, Zn, Mo not detected.

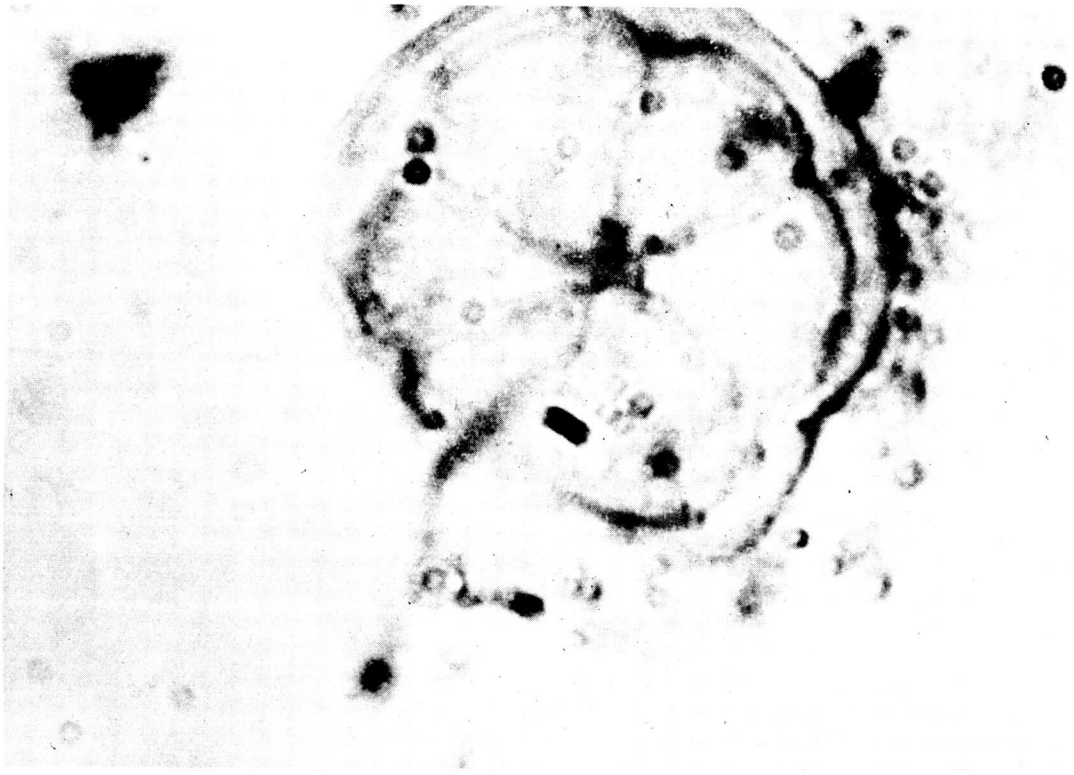


Figure 8 Typical Kakabekia–like form grown on nutrient agar in 50% NH_3 + 50% air. Photographed at 950X.

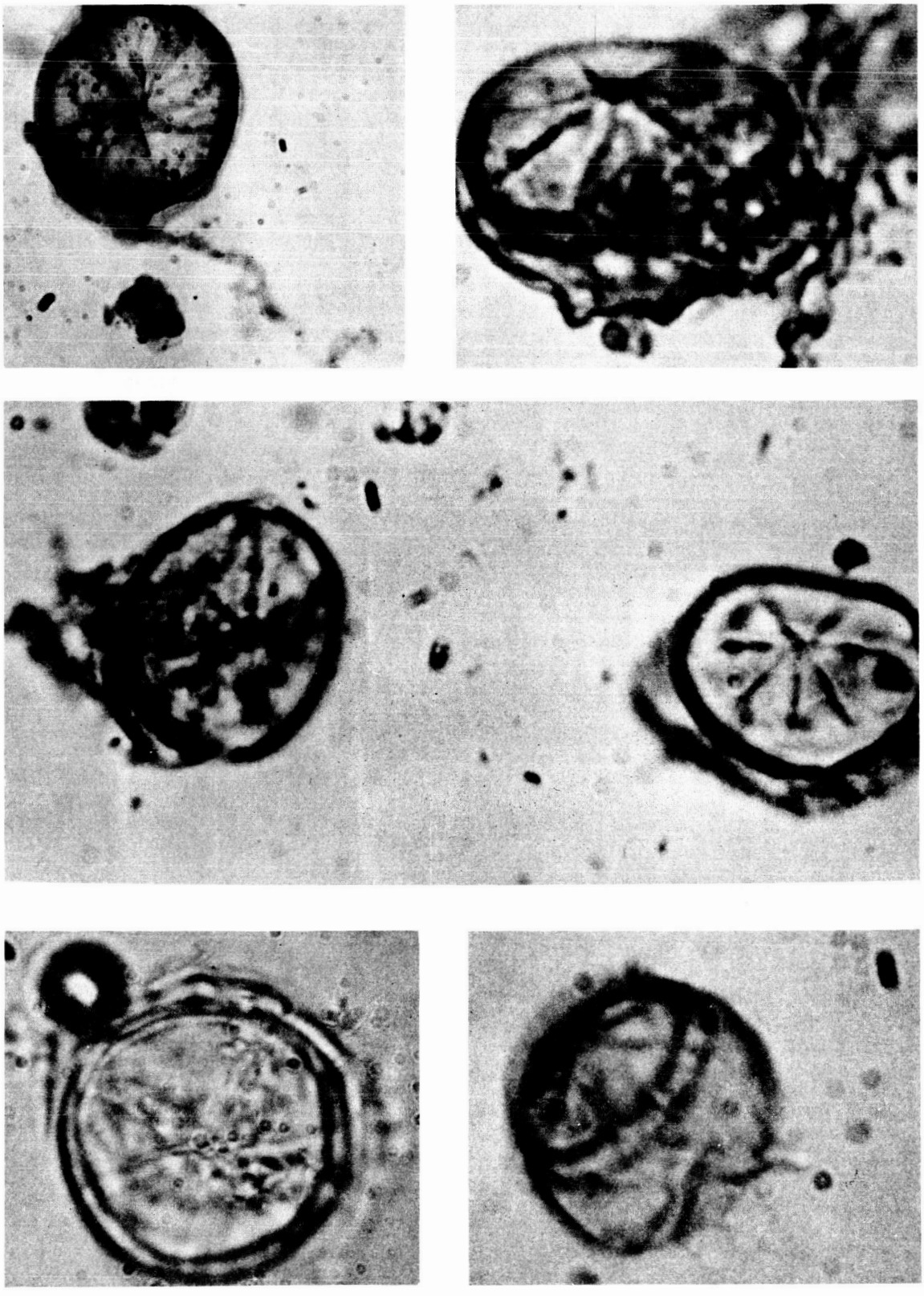
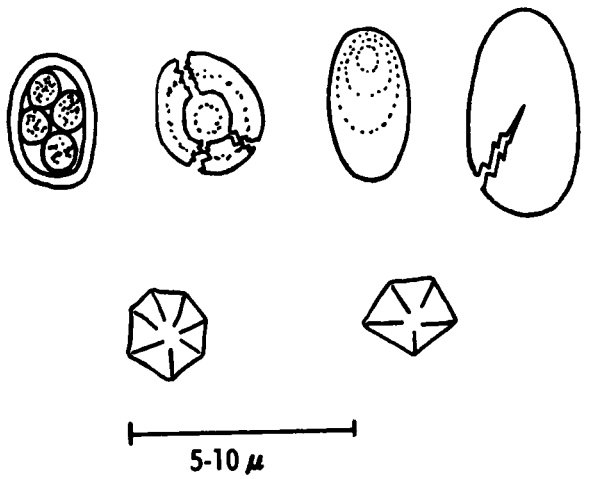


Figure 9 Morphological variations in the Kakabekia–like forms grown on agar under ammonia–air atmospheres. Photographed at 950X.



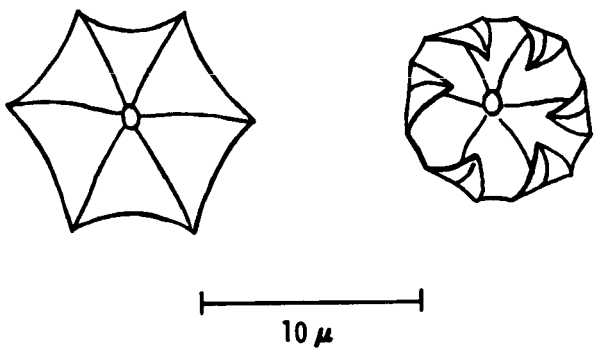


Figure 10 Recognition problems in the examination of cultures for Kakabekia–like forms.
Above: Diagrammatic representation of confusing structures.
Below: Diagrammatic representation of typical and in–folded Kakabekia–like organisms.

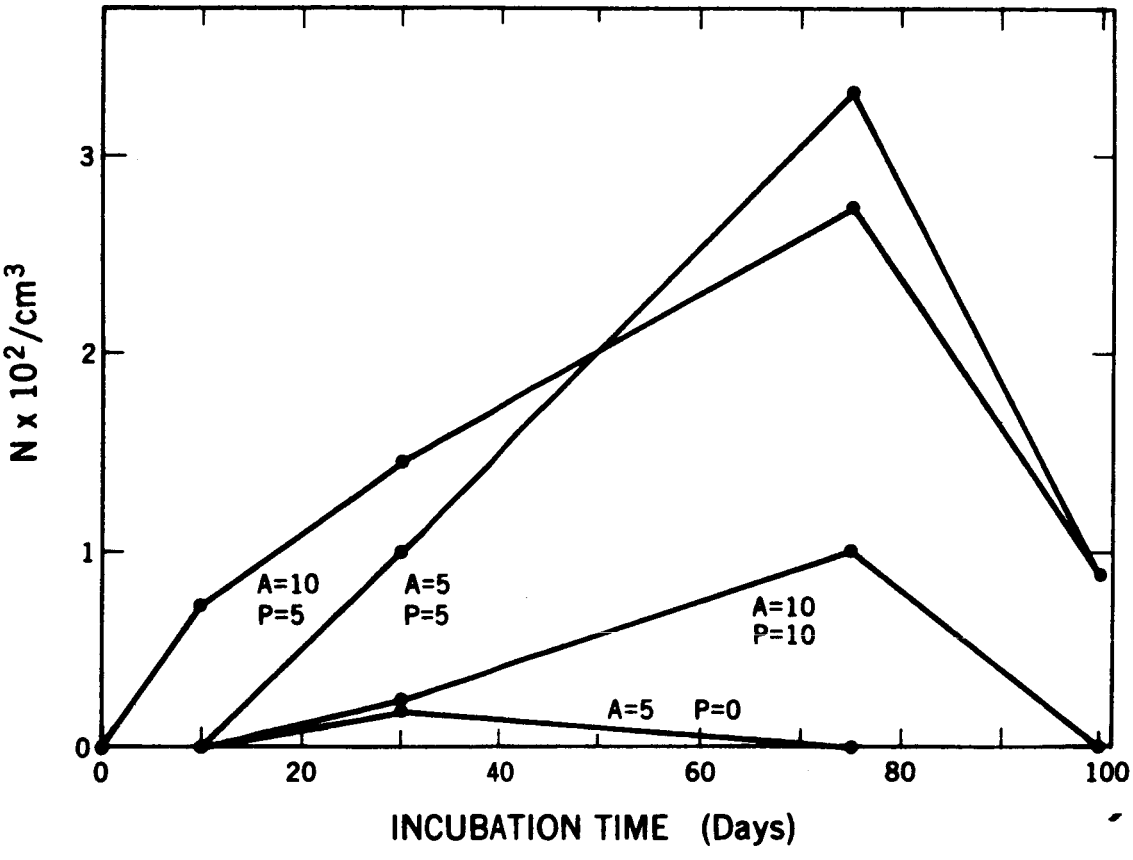


Figure 11 Appearance of Kakabekia-like forms in glucose-ammonia-peptone broth inoculated with soil Sample H-1. Glucose present at 10 g/l, ammonia ("A") at 5 or 10 M, and peptone ("P") at 0 or 5 g/l.

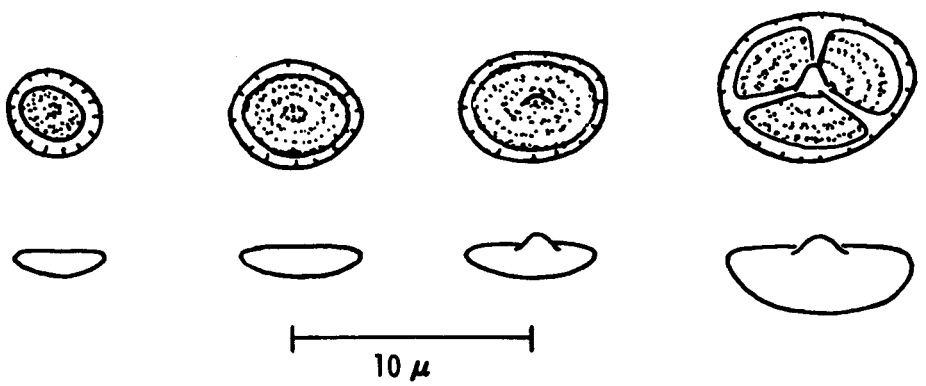


Figure 12 Diagrammatic representation of a possible ontogenetic series.

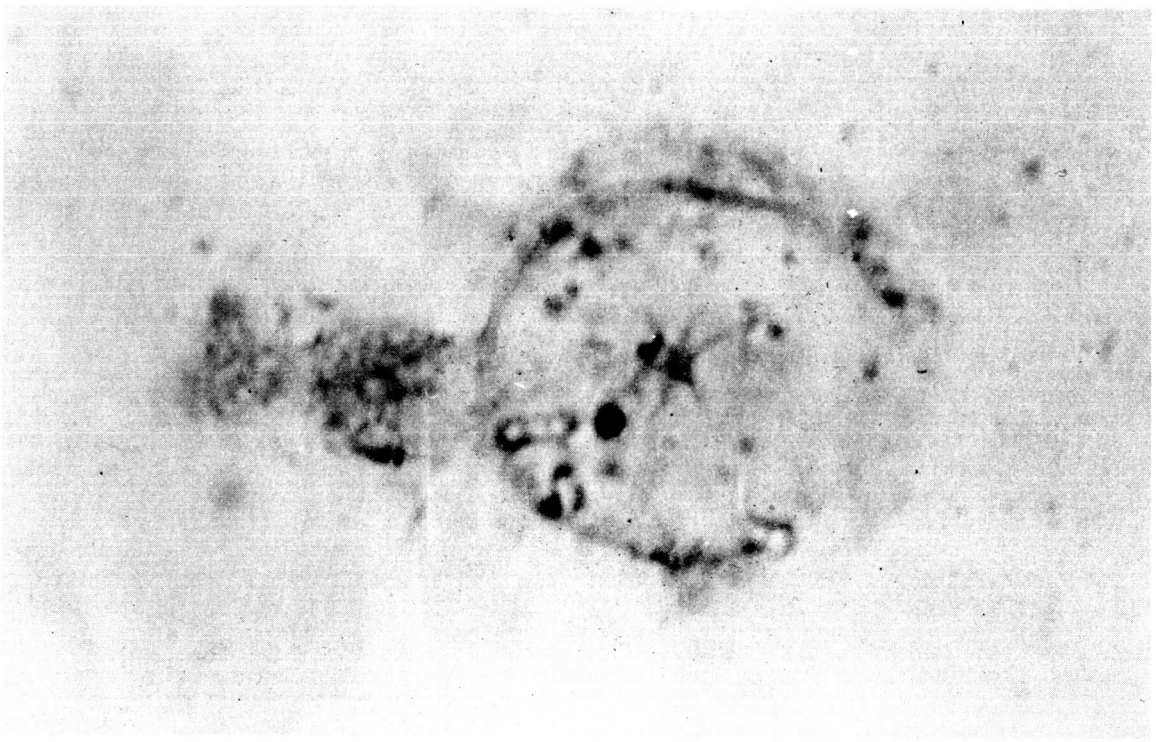


Figure 13 Degenerating (senescent) Kakabekia–like form from a 6–week culture. Photographed at 1250X.

V. NOBLE GAS ANOXIA

A. Studies on the Mechanism of Differential Lethality of Chemically Inert Gases

Rates at which seeds of Prolific rye and Genessee wheat lose viability in anoxia are inversely correlated with molecular weights of chemically inert gases used to induce anoxia. Mortality of oats and rice subjected to similar conditions is unaffected by atmosphere (Semiann. Rept. 1, November 1966 Section IV pp. 79-82). One possible explanation of the differences produced by chemically inert gases is that rye and wheat respond to a decrease in weight of molecules comprising the ambient atmosphere as they would to an increase in temperature. Two experiments have been conducted to test this hypothesis.

These experiments utilized two glove boxes maintained at 22°C and 30°C respectively. Four helium-tight anaerobic chambers each of which contained 100 seed samples of Prolific rye, Genessee wheat, Vicar oats and Caloro rice, were placed in each glove box. Two of the small chambers in each glove box contained helium, two were filled with nitrogen. The glove boxes were purged continuously with nitrogen during treatment.

Exposure to 12 days of anoxia at 22°C elicited typical responses from each of the four genotypes. The helium sensitive species, rye and wheat, showed a 203 fold greater loss of viability in helium than in nitrogen (Table 22). Oats and rice characteristically showed no differential responses to these atmospheres. At 30°C anaerobic stress of similar duration and intensity produced markedly greater losses in viability of rye, oats and rice. However, wheat, the species usually least tolerant of prolonged anoxia at 22°C, survived nearly as well at 30°C and showed the same differential response to nitrogen and helium.

These interspecific differences in response tend to preclude acceleration of events leading to loss of seed viability as a universal effect of increased temperature, within the range of physiological tolerance of the organism, during treatment. Further, if the differential sensitivity of rye and wheat to nitrogen-and helium-anoxia were due to simulated effects of higher temperature in the lighter gas, elevation of temperatures should have produced a proportionally greater increase in mortality of seeds exposed to nitrogen than among those seeds held in helium. Although the response of rye was consistent with this hypothesis, that of wheat was not. The consistency in performance of wheat at widely different temperatures indicates that its response to anoxia and to different anaerobic environments was governed by something more than rate differences produced by increased temperature or analogous physiological effects of helium.

B. Comparative Lethality of Hydrogen and Chemically Inert Gases

Effects of Hydrogen-anoxia on viability of hydrated seeds differ quantitatively but apparently not qualitatively from effects of the chemically inert gases. Results of some recent comparative tests (Table 23) are consistent with previous findings that for those seeds sensitive to differences in anaerobic atmospheres, i.e., rye and wheat, rates of decline in viability are inversely correlated with molecular weights of the ambient gases. Both hydrogen and helium are more deleterious than is nitrogen. Although the two lighter gases do not differ markedly or consistently in their effects in this instance, seed viability often declines somewhat more rapidly in hydrogen than in helium.

Table 22. Effect of Temperature on Loss of Seed Viability During 12 Days Exposure to Nitrogen-and Helium-Anoxia. Percent Surviving After 21 Days' Recovery in Air.

Temperature	Gas	Rye	Wheat	Oats	Rice
22°C	Air	91	92	79	97
	Nitrogen	24	33	42	47
	Helium	11	12	44	48
30°C	Nitrogen	1	27	8	1
	Helium	1	6	3	0

Table 23. Comparative Lethality of Nitrogen-, Helium-, and Hydrogen-Anoxia at 22°C. Percent Surviving after 12 Days' Exposure to Anoxia and 21 Days' Recovery in Air.

Atmosphere	Rye	Wheat	Oats
Air	93	24	81
Nitrogen	25	23	44
Helium	15	10	49
Hydrogen	11	10	47

VI. RESEARCH HIGHLIGHTS: 1963-1967

Our program under contract NASw-767 was inaugurated with the implicit belief that any consideration of extra-terrestrial environments suitable for grossly familiar life must first take into account the ecology and bio-geography of the Earth integrated over bio-geologic time. The phrase "the Martian Environment" has about as much meaning as "the Terrestrial Environment". Although a long-lived organism may encounter a fairly large proportion of its total planetary environment in one form or another, given a period of thousands of years, this contact is quite indirect. In the final analysis, the fate of the organism and status of its existence are bound up with the microhabitant and, in the writer's opinion, all aspects and considerations of evolution and ecology must be referred to immediate surroundings and conditions and their changes and progressions.

Most of the collected environmental parameters of Mars are readily observable in terrestrial microhabitats. Often combinations such as low P_{O_2}-low temperature (alpine) or low P_{O_2}-low net g (aquatic/marine) exist prominently. Low net g-low P_{H_2O} may be found in brine pools and pounds at saturation. Don Juan pond in Antarctica is of most interest as a near-saturated calcium chloride-calcium chloride hexahydrate solid phase. Water activity of this pond is ca 0.35.

In the early 1960's in spite of the common assumption that the anaerobic bacteria were the only serious candidates for a Martian flora, we took the viewpoint that the evolutionary experience of a differentiated multicellular terrestrial organism could have advantages over some of the intensified versions of terrestrial stresses to be found on Mars. Seeds, vascular plants and invertebrate animals were, accordingly,

tested for their capabilities against variations in a series of environmental parameters--atmospheric pressure and composition, water, temperature, thermoperiod, radiation and toxicants.

A sufficient justification for performance trials with complex organisms, especially those adapted to drier terrestrial locations, may be found when one examines the events that take place when ordinary well-watered habitats shift through geo-climatic changes, toward the xeric condition. Any sufficiently large local population will harbor a large proportion of obligate mesophytes now doomed to extinction, relieving competitive pressure upon more xerophytic plant forms. Such plants, often with small leaves or photosynthetic stems, held in check in a dense mesophytic population by shading as well as crowding, take over from their less hardy predecessors. As xeric conditions intensify, selection will increase frequencies of "desert-hardiness" genes, especially if those plants in the increasingly arid location are isolated in terms of ready interbreeding (cross-pollination) from their more mesophytic relatives. Our trials have been far more severe, of course, but the plants tested have commonly been species already selected in nature for stress tolerance. Nevertheless, the challenge is presented without transition to withstand anoxia, night temperatures of 243-353°K, atmospheres with dew points of ca-210-220°K, and the combination of these three conditions. Seed trials were less stringent in the sense that a moderate quantity of initial water had to be supplied. Nevertheless, only a few hundred mg/cm^2 were required for the outstanding species. To be sure, it is impossible to know whether there is sufficient water on Mars to meet the needs of terrestrially-derived plants. If Mars were historically less of a desert planet than

we now think it to be, then the long period of water loss that must have taken place should have provided ample time for selection to reveal any latent high-efficiency water concentration mechanisms. One of the simplest and most obvious ways to accomplish this is to develop a bio-concentration mechanism based upon hydroscopic salts. (Some acetates, sulfates, nitrates and halides are possiblities.) The results of test for terrestrial capabilities showed that a few seeds such as rye and rice can product seedlings under our gross simulator conditions. Air-grown plants, including cacti of the genius Cereus, and conifers of the genus Pinus, and a few others retain their structural integrity and biochemical activities (including photosynthesis) after 1-10 months in the simulator. One plant, Haworthia, died in a few days, but produced vegetative shoots which grew in the simulator. In all, over 300 species of plants have been examined for stress tolerance, and perhaps five per cent display capabilities that would qualify them as nearly "Mars-adapted" vegetative organisms. Conceivably a few years' selective breeding could close the gap if it were not for one critical limitation: the flowering process in terrestrial plants, unlike its vegetative processes, is highly aerobic. Specifically, bud opening requires a minimum of ca 5-10mm O_2 and is rate-limited up to oxygen pressures of ca 38 mm. Furthermore, atmospheric conditions believed to exist on Mars would not favor pollination, by wind or earth-like insects (the aerodynamic design of bees and similar insects is not suitable for the low density of the Martian atmosphere). It seems at this stage to be a safe generalization that adaptability of terrestrial vascular plants to the best defined extra-terrestrial environments resides in vegetative juvenile structures.

Important technical by-products of these studies have appeared at several levels. First, there is an interaction between freeze-damaged plant cells and oxygen that renders mechanical damage non-reparable Cold-sensitive species such as cucumber, dwarf palm and boxwood can be rendered more freeze-resistant if they are either grown at sub-atmospheric O_2 levels or subjected to freezing and thawing in 5% O_2 or less. Second, the chemical composition of plants can be regulated without disturbance to growth by cultivation at sub-atmospheric O_2 levels. Generation of unusual and hazardous metabolites such as H_2 and CO have also occurred. Third, (already indicated), juvenile tissues are more stress tolerant. Fourth, the first outlines of the developmental role of molecular oxygen in plant ontogeny have been laid down. Fifth, as a logical exercise, it seemed reasonable that the microaerobic capabilities evidenced by seeds should permit them to germinate in aerated water. This expectation was fully confirmed, and it now seems possible that some land plants can adjust to submerged culture in saline waters, even full-strength sea-water, if adequate aeration is maintained. Highly adapted desert species also can survive long submergence (ca 10 months), suggesting that the "goodness-of-fit" aspects of the evolutionary niche concept have been over done. Finally, unique effects of helium anoxia have been demonstrated with plants and the viability-sapping properties of helium as a second gas compared with nitrogen have been shown using HeLa cells.

In "The Atmospheres of the Earth and Planets", Kuiper uses lichens as a "heuristic model" for Martian life. Further the conclusion that "lichens and beetles" are examples of the highest Martian life is attributed to him. Among some two score invertebrate species studied,

the larvae of the beetle Tenebrio are strikingly adapted to simulator conditions. After 70 consecutive days including exposure to 12 hr "nights" of ca 240-250°K (and dry anoxic atmospheres) these mealworms were still capable of normal-appearing activity. Other arthropods--beetles, ants, spiders--have been studied only as adults, and although they exhibit high degrees of stress tolerance, they do not approach that of the Tenebrio larvae.

The growth of the native lichen of the tundra, Cladonia rangiferina has not been studied because it takes place too slowly. However, its respiratory activity and stain permeability together have been used as an index of viability. On the basis of these characteristics, its metabolic capabilities are retained at 233°K in saturated LiCl. Sub-freezing LiCl yields a water activity $\ll 0.01$, an "ultra-dry" liquid environment by any standards. On the other hand, desert lichens on rocks have long been recognized for their tolerance toward temperatures $\gg 373$°K. They are of course dormant when exposed to such "protein-cooking" temperatures. It would be a surprise if lichen samples placed upon the surface of Mars were to perish, even after an indefinite exposure. This position includes not only consideration of the atmosphere-temperature-water complex, but ultraviolet radiation as well. The principal factor of uncertainty is high energy radiation, but radiation "hardening" is as much a terget for genetic selection as cold tolerance, hence the extent to which ionizing radiation is harmful on Mars relative to its ability to increase mutation frequency (i.e. opportunity for innovation) is a matter of conjecture.

Ultraviolet resistance is not unique to lichens. Indeed, in the absence of O_2, ultraviolet radiation is a questionable hazard to multicellular organisms. Such varied forms as seed plants, spiders and turtles can withstand Martian surface ultraviolet level at least for weeks.

Ultraviolet shielding is a "built in" property of differentiated plant and animal bodies possessing integuments. Other more defenseless forms may be protected by shielding behind rocks, in crevices, etc. It is not beyond conception for plant-like forms to carry on UV-photosynthesis when anoxia precludes destructive photo-oxidation.

The majority of organisms tested during this inaugural, heavily Mars-oriented phase of the program failed the most rigorous treatment, although remarkable capabilities were repeatedly exhibited. Some, such as the turtle, were not seriously examined as an extra-terrestrial model, but, rather to find out how far from the environmental norm a complex animal could be taken. These animals were invaluable because they destroyed one conception that had been held previous to their introduction. It was long assumed by the writer that organisms with microaerobic capabilities were relatively intolerant toward high oxygen partial pressures. However, the turtle was shown to live normally at P_{O_2}=15 mm and at P_{O_2}=760 mm for many days. It was evident that the tolerance of individual species could be extremely wide in range with respect to O_2. Of course, sufficiently long-term exposure of turtles to P_{O_2}=760 mm resulted in lung pathology and death. Within limits, compounding O_2-stress by pressures of up to 7 atmospheres or intense ultraviolet revealed still more remarkable resistant factors in these reptiles of ancient lineage. The turtle eye seems to possess amazing resistance to ultraviolet radiation, although grossly it is a typical vertebrate organ. This resistance alone could become a substantial subject for investigation.

Second only in importance to ultraviolet tolerance, was the demonstration that a free-living (ex ovo, here) vertebrate could sustain activity without cardiovascular function. In general, during periods of up to 4 months at P_{air}=75 mm. the total blood volume of 7 gram turtles

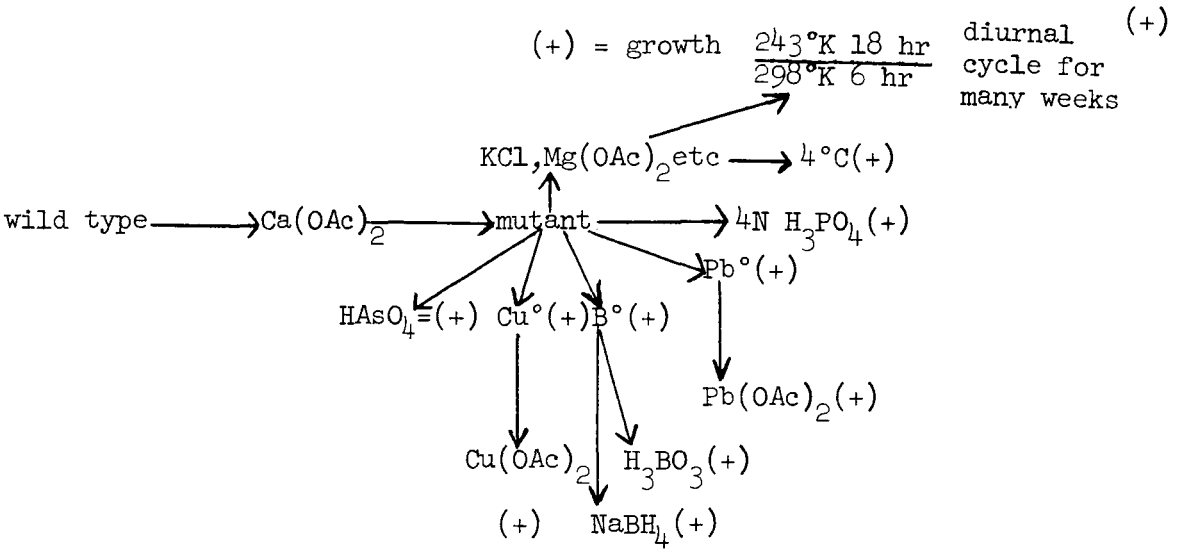
fell from ca 0.5 cubic centimeters to about one-fifth that volume. An exception was one animal caught swimming that yielded no vascular fluid and, upon total dissection, was found to be devoid of circulating fluid. Carbon monoxide tolerance (75% CO + 5% O_2 + 20% N_2) for 6 hrs suggested that specimens of this size can be supported in O_2 gas exchange purely by diffusion, independent of hemoglobin.

Although it is fair to say that maximum stress tolerance was defined for many species, and could be resolved frequently with respect to O_2, temperature, water or combined factors, most moribund organisms were characterized by necrotic lesions rich in microflora. Fusarium, Neurospora and Alternaria, growing upon dying conifers or succulents in the simulator, began to be of interest in their own right. When the original position with reference to the aerospace-exobiological capabilities of higher forms became more secure, an active investigation of the lower forms was initiated. The predominantly microbiological phase of this program thus began with the demonstration that fungi could achieve what vascular plants could not--a complete life cycle(from spore to spore) under extreme stress environments. Sporulating mycelium on dead conifers, cacti, ivys, hollies, etc. was commonly associated with almost totally desiccated organs and tissues in the extremely dry simulator atmosphere. This fact, combined with the performance of the lichens in salt media, not only introduced the possibility of a novel major microhabitat on Mars but also carried the concepts of survival in hostile environmentals to new extremes. In essence, if it is accepted that Mars passed through a relatively substantial organic atmospheric phase complete with erosion, downgrading and chemical decomposition of primary minerals and rocks, then there should have been at some point shallow seas of increasing salinity. As such saline bodies

shrank, they would have left (according to topography) various forms of brine pots and pools, containing Group I and II halides. At saturation, a $CaCl_2$ brine should provide an all-season stable aqueous liquid phase for living matter with the added features of serving as a powerful ultraviolet screen and of being a sufficiently dry medium to float limonitic dust without wetting it. With the subsequent demonstration of a Penicillium mutant capable of growth in all the acetates and chlorides of Na, K, Mg and Ca, and of bacteria and other microflora growth in lithium salts, the case for suitable microhabitats for life on Mars is deemed to be advanced as far as possible.

As the exploratory goal was moved from a fixed planetary objective toward a broad attack on the question of life under extremes, all previously successful adaptations became relative failures. It was decided to step up the selection pressure using the mutant Penicillium.

Some of the stress factors to which it has been subjected were as shown:



Other stress factors involved the use of non-aqueous solvents, including acids, alcohols, formamide, etc., combined with water in varying proportions, and liquid NH_3 at 233°K. Other fungi with growth

capabilities in NH_3 atmospheres/NH_3 (aq.) media were disclosed as the work proceeded and a true "ammonophile" identical with Kakabekia umbellata Barghoorn, a Precambrian fossil form (ca 2 x 10^9 yrs) was discovered in soil from Wales. Again, limits of adaptation have not yet been reached, hence the program must be pursued. By chance a case for life on Jupiter was made out of tolerance to the system NH_3(gas)/H_2O(liq) plus evidence for growth and retention of vital function in liquid NH_3 at 233°K.

Although the use of aqueous ammoniacal systems seemed at first to permit a reduction of experimental materials strictly to the microbiological level, the curious capability of onion and other members of the genus Allium to germinate under 760mm NH_3 once again demonstrated that even severe toxicants cannot eliminate multicelluar life altogether.

Obviously, the success of this program lies in its failure to locate and define the extreme limits for terrestrial life, a consequence of the persistance of life itself.

During the four years that this contract has been in force, 19 papers have been published in Icarus, Proceedings of the National Academy of Sciences, Botanical Gazette, Plant Physiology, Nature, Science, Aerospace Medicine, Proceedings of the Tenth Internation Botanical Congress (Edinburgh, 1964) and Proceedings of the 6th International Biochemical Congress (New York, 1965). Major review articles have been published in Current Aspects of Exobiology (Eds. Briggs and Mamikuniam, J.P.L. Publication) and Advances in Space Science and Technology (Ed. Ordway, In Press 1967). A semi-popular review is in press in Encyclopedie De L'Space (1967)

An additional six technical papers are now in preparation or have been submitted for publication.

Six papers not issued otherwise were read at meetings of the Institute of Food Technologists (Washington, 1964), Southeastern Section, ACS (S. Charleston, 1965) and American Institute of Biological Sciences (Univ. Illinois, 1965, Univ. Maryland, 1966).

Invited seminars were given at UCLA in 1964 and 1965, the University of Massachusetts, 1964 and 1965; Brooks AFB (School of Aerospace Medicine 1965, 1966; Manhattan College, 1966; Rutgers University, 1966; Lamont Geological Observatory of Columbia University, 1966; and the State University of New York at Buffalo, 1967.

Finally, talks have been given to nearly ten thousand elementary and high school students and teachers in New York and Ohio on the subjects of environmental biology and exo-biology.

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