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PHOTOSYNTHETIC HALOPHILES FROM OWENS LAKE

by R. W. Tew

Prepared under Contract No. NASw-1037 *by*
SPACE-GENERAL CORPORATION
El Monte, Calif.
for

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION - WASHINGTON, D. C. - JANUARY 1966

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ABSTRACT

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R. W. Tew

Studies of growth limitation by water have been accomplished with halophilic, anaerobically photosynthetic bacteria from an halite-thenardite-trona evaporite deposit in the Owens Valley. A purified isolate has been identified as a member of the group of small Chromatium species promoting sulfur formation in the growth medium. The optimum a_w for rapid growth of this organism appears to be .95. In solutions of NaCl or of sodium carbonates at pH 9.5, growth is a function of a_w; in sodium sulfate brines the response is also related to a property of the solute. Generation times were 78 and 13 hours in media having water activities of .76 and .95. Growth has been studied in biphasic systems in which the concentration of more than one solute, water activity, and the composition of the solid phase were predicted by phase rule. Organisms trapped within mirabilite crystals during these experiments survived liquid nitrogen temperatures and subsequent exposure to vacuum. Initial results of a theoretical study of halophilic growth indicated that, in the saturated solutions used, water does not move osmotically, but must be pumped into the cell.

In a discussion of the practical significance of this research, the importance of geochemical characterization of the environment as a prerequisite to detection of life by detection of growth has been emphasized. The importance of phase transitions of hydrated to anhydrous salts has been noted, and postulated as a possible temperature controlled mechanism for the release of water for the growth of halophiles.

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Section 1

INTRODUCTION

This is the final report of progress on "Photosynthetic Halophiles from Owens Lake," and is submitted in accordance with the requirements of Contract No. NASw-1037.

Two of the ways in which the Martian environment differs significantly from Earth are the scarcity of free water and the absence of atmospheric oxygen. This implies that Martian life must either occupy an occasional niche in which free water exists, or must concentrate water from sources in which, by terrestrial standards, it would be tightly bound and unavailable for life. On Mars, energy for synthesis and for the accumulation of water for synthesis would of necessity be derived from an anaerobic process, although, for thermodynamic reasons, being ultimately dependent upon the sun.

Studies of growth limitation by water or anaerobic photosynthesis under conditions in some way approaching Mars may be accomplished with a number of terrestrial organisms from a number of environments. However, only in rare cases can both be investigated simultaneously. The literature is replete with references on halophiles and on photosynthetic bacteria, but is notably lacking in publications on photosynthetic halophiles. Important exceptions are References 1 and 2 on photosynthetic and sulfate reducing bacteria in the Wadi Natrûn, and publications of Van Niel[3] and Baas-Becking[4] which report results of studies on halophilism within 1) the genus Chromatium, and 2) Thiorhodaceae from Searles Lake of the Owens River chain and from Owens Lake itself.

Characterization of photosynthetic halophiles and their Owens Valley environment has been an intermediate objective of research discussed in this report. These studies have been carried out within the more general frame of reference of the program which is concerned with the identification of mechanisms permitting the existence of both photosynthetic and sulfate reducing halophiles in the saline environment, and the extent and energetics of water

limitation of growth of the photosynthetic species on salt crystals and in brines. The approach was to bring biological and geochemical data together by explanations founded in thermodynamics.

Section 2

MATERIALS AND METHODS

Methods used routinely throughout the investigations are listed here. Special procedures required only occasionally are given in the appropriate context in the individual sections of the report.

The nutrient solutions for studies in solid and liquid media are defined in Table 1. Analytical grade sodium chloride, Na_2SO_4 $NaHCO_3$, Na_2CO_3, and commercial grade $NaHCO_3 \cdot Na_2CO_3 \cdot 2H_2O$ were added as required for experiments with artificial brines. Solid media for isolation and maintenance of isolates contained 60 grams NaCl, 20 grams $NaHCO_3 \cdot Na_2CO_3 \cdot 2H_2O$, 35 grams Na_2SO_4 and 15 grams of agar per liter. Brines were sterilized by pasteurization for 1 minute at 85°C after filtration through 0.45 micron sterile Millipore filters. When solid salt phases were added, pasteurization was the only treatment used. The agar component of solid media was autoclaved separately and added to pasteurized brine at 45°C.

Cultures were routinely incubated under 2450 fc illumination from 100 watt fluorescent lamps, which does not provide an optimal spectral distribution. Growth temperatures were 25±1°C, or 32±1°C.

Experiments with liquid media were carried out in 50 ml Erlenmeyer flasks containing 50 ml brine and provided with rubber stoppers. Pasteurization, nitrogen purging, and the H_2S reaction with oxygen were relied on to create conditions of anaerobiasis and low oxidation reduction potential. Nitrogen purging probably did not reduce oxygen tensions appreciably in the gaseous phase over the cultures. Enrichment cultures were carried through serial transfers in milk dilution bottles filled nearly to the rim, pasteurized, again filled to the rim with pasteurized medium, inoculated, and capped.

Growth was evaluated by determination of optical density at 525 and 780 mμ and by cell counts (Petroff-Hauser). Optical density data were taken by use of 1 cm round cells in a Bausch and Lomb Spectronic 20 spectrophotometer, for which a red sensitive photocell was provided. Values over 0.500 were obtained by dilution, optical density determination, and multiplication by the dilution factors. Generally, cultures were cut in a Waring blender prior to analysis. This method has the disadvantages of being dependent on pigment synthesis, which may vary with cultural conditions and of interference from sulfur formed in the medium. Oxidation reduction potentials and pH were determined with a pH meter, and corrected for sodium ion concentration when necessary.

Chemical analyses were performed according to methods in Reference 5, as follows: Fluoride, Megregian-Maier method; chloride by titration with mercuric nitrate; sulfate by the turbidimetric $BaSO_4$ procedure; carbonate by electrometric determination of titration curves with .02N N_2SO_4; and sulfide by the methylene blue procedure. Computations of molalities were dependent on measurement of specific gravity by weight or hydrometer. Potential interference

by accompanying ions exerted an important influence over the choice of sample sizes and aliquots.

Statistical quantities (means and standard deviations) indicated in the tables were derived from analyses of at least four separate cultures.

Table 1

Composition of Nutrient Solutions

Component	Grams/Liter	
	Solid Medium	Brines
NH_4Cl	.4	1.0
$K_2HPO_4 \cdot 3H_2O$	2.0	5.0
$MgSO_4 \cdot 7H_2O$	.2	1.0
$Na_2S \cdot 9H_2O$	4.8	2.4
$Na_2S_2O_3$	4.0	5.0
Fe	.001*	.0001*
Minor Elements**	0.1 ml**	0.1 ml**

* as Fe citrate chelate

** Hutner's trace elements

Section 3

RESULTS

3.1 THE ENVIRONMENT

The particular environment of concern is an evaporator pond in the Owens Valley, which has a total salinity of 346,000 parts per million and a brine pH of 10.3. Owens Valley is a closed basin which lies between the Sierra Nevadas, and the Inyo Mountains. The floor of the valley was formed by subsidence along parallel faults and was concurrently filled to a considerable depth with alluvial debris from the surrounding mountains and with the results of extensive tertiary of early quaternary vulcanism[6]. Unequal subsidence and partial rotation of the fault blocks of the valley floor resulted in the formation of the depression presently occupied by Owens Lake.

Until approximately 4,000 years ago, Owens Lake was part of an open, or exorheic drainage system, which included, among other presently closed basins, Death, Panamint, and Searles Valleys[7]. At that time declining rainfall and glacial retreat combined to bring about separation of Owens Lake from the rest of the system. Owens Lake became an area of closed, or endorheic drainage, potentially susceptible to accumulation of salts.

The analyses of Owens Lake reported in 1905[8] (Table 2), 33 years after diversion of the Owens River into the Owens Valley irrigation system, reflect the progressing extinction of the Lake. Since 1913, when the Los Angeles Aqueduct was put into operation, Owens Lake has dried to the point where extensive deflation by frequent high winds is in progress.

The evaporator pond (Vat Number 4) is located at Bartlett, California, on the property of the Pittsburgh Plate Glass Company. A comparison of the evaporator pond, the former Owens Lake, the lakes in the Wadi Natrûm, and other salines appear in Tables 2 and 3, and emphasizes the extremely concentrated nature of the environment under study. Evaporation rates are given in Reference 6.

Table 2

Comparison of Saline Waters by Class and Composition

Name	Class	Percentage Composition of The Total Salts Present							Salinity, p.p.m.
		Cl, Br	SO_4	CO_3	Na, K	Ca	Mg	B_4O_7	
Evaporator Pond	Triple	30.02	9.15	17.3	39.2	-	-	3.96	346,000
Owens Lake, 1886	Triple	25.09	9.73	25.16	36.96	0.02	0.03	-	77,100
Owens Lake, 1905	Triple	32.31	9.93	24.55	39.62	0.02	0.01	-	213,700
Wadi Natrûn, Lakes	Triple	4.0	45.5	15.3	35.2	-	-	-	180,000 - 239,000
Dead Sea	Bittern	68.55	0.28	Trace	13.04	4.26	13.90	-	20,000 - 25,000
Great Salt Lake	Chloride	55.64	6.52	Trace	35.43	0.34	2.22	-	204,000
Borax Lake	Carbonate-Chloride	32.31	0.13	22.47	39.62	0.03	0.35	5.05	76,000
Caspian Sea	Sulfate-Chloride	41.96	23.88	0.65	25.16	2.44	5.87	-	12,900
Moses Lake (Washington)	Carbonate	3.88	2.88	2.87	51.56	19.86	8.41	7.25	2,966

Table 3

Composition of Evaporator Pond Effluent Brine*

Date	Air Temperature 7:00 AM	Air Temperature 3:00 PM	Specific Gravity	7:30 AM Brine Temperature	% By Analysis Na_2CO_3 ± 0.10	% By Analysis $Na_2B_4O_7$ ± .0001	% By Analysis NaCl ± .001	% By Analysis Na_2SO_4 ± .005
5-30-65	59	91	1.318	69	10.88	1.96	16.70	5.36
29	58	93	1.319	70	10.67	2.00	16.69	5.36
28	46	90	1.319	69	10.58	1.96	16.72	5.50
27	60	86	1.316	61	10.29	1.91	16.78	5.76
26	58	83	1.315	63	10.03	1.88	16.78	6.02
25	-	-	1.319	-	9.76	1.87	16.86	5.68
23	51	72	1.313	55	9.75	1.81	17.11	5.62
22	45	74	1.318	58	10.42	1.85	16.49	5.72
21	62	82	1.318	60	10.62	1.82	16.58	5.48
20	62	86	1.320	62	11.12	1.72	16.42	5.44
19	58	92	1.283	64	12.51	1.70	15.30	5.42
18	61	91	1.316	65	10.38	2.00	16.19	6.14

* Data Courtesy of Pittsburgh Plate Glass Co., Bartlett, California

The evaporator pond has an area of 22 acres and an average depth of 3 feet, or, is of sufficient volume to contain 21.5 x 10^6 gallons of brine. Salt deposition reduces the potential liquid volume; during the winter of 1964-1965 near-surface deposits were 80% salts. Brine from a well in Owens Lake proper is pumped into the pond from May to October at a rate of 250 gallons per minute (plus compensation for evaporation) and is removed at the same rate. The pond is thus analogous to a chemostat. If no deposited salts were present, the entire volume of the pond would be replaced in 60 days. With 50 and 80% of the volume occupied by solid salts, an organism must have a generation time less than 30 or 12 days (including lag phase) to achieve an increase in number. This calculation does not account for the limit of light penetration, therefore is probably a conservative estimate. Obviously, if the dilution rate exceeds the possible generation time at prevalent a_w, growth of the photosynthetic bacteria must be confined to a solid, illuminated surface, or to any area where flow is obstructed by deposited salts. Here, the total growth possible becomes a function of light, substrate and dilution rate; the growth rate will be dependent upon water activity and temperature, as long as light and substrate are not limiting.

Therefore, it seems significant that the microenvironments are apparently layers in the deposited salts which are easily differentiated by their pink color. These layers are isolated from the air interface by a superimposed layer of white crystals, are often rather deeply pitted, and are separated from the bottom of the pond by a second layer of white minerals.

Samples of salts, brine, and mud from the bottom of the pond were obtained on 27 July, 19 August, 5 October and 31 October 1964. Although the white superimposed deposits varied in thickness and extent over this sampling period, there was little change in the appearance or location of the pink layers.

From 29 November 1964 to May 1965, the evaporator pond was a solid mass of salts with no surface brine in evidence. In appearance, the pond surface was strongly reminiscent of photographs of arctic permafrost with well defined polygons of about 15' diameter demarcated by pressure ridges. Near the edges of the pond, the white surface layer of salts was subtended by a layer of purple,

very hard material. Near the center of the pond, this purple layer was present only beneath the surface of the ridges or areas immediately adjacent, and was of a slushy consistency. The pond in its winter condition is illustrated in Figure 1.

Flooding was initiated on 16 May 1965. On July 4, 1965, approximately 1/3 of the surface was covered with brine to a depth of one to three inches, as it was during the same month of the previous year.

The concentration of sulfide in the brine is given in Table 4. Apparently, the brine source in Owens Lake proper is the source of sulfide for the pond. The proportion of sulfide originating from the activity of Desulfovibrio species in the Lake sediments and from water liberated from or contacting igneous intrusions is not known.

Composition of the Solid Phase

The salt cake samples obtained 27 July and 19 August were analyzed by X-ray diffraction (Figures 2 and 3). In general, the purple or "growth" layers of samples of salt cake appeared to consist of thenardite (Na_2SO_4), sulfohalite ($2Na_2SO_4 \cdot NaCl \cdot NaF$), trona ($NaHCO_3 \cdot Na_2CO_3 \cdot 2H_2O$) and a trace of halite (NaCl). The superimposed white layer from the 27 July sample contained the same minerals, but proportionally much more halite; similarly appearing material from the 19 August sample contained over 90% halite, a trace of thenardite, and no trona or sulfohalite. Burkeite, $2Na_2SO_4 \cdot Na_2CO_3$, was anticipated but not found in any of the samples.

A second triple salt, gailite ($Na_2SO_4 \cdot NaCl \cdot NaF$) was thought to be present in the growth layers of the salt cake. The postulated existence of this salt and closely related sulfohalite ($2Na_2SO_4 \cdot NaCl \cdot NaF$) in the salt deposits was remarkable in that it implied high concentrations of NaF in the brine.

A phase rule approach was applied to determination of the environmental conditions promoting the deposition of the particular combination of salts found in the evaporator pond. This information was important for simulation of the pond environment in the laboratory and determination of water activity. Diagrams of the systems (1) $NaCl-Na_2SO_4-Na_2CO_3-H_2O$, and (2) $NaCl-NaHCO_3-Na_2CO_3-H_2O$ are presented in Figure 4, the reader is actually looking on the structures from

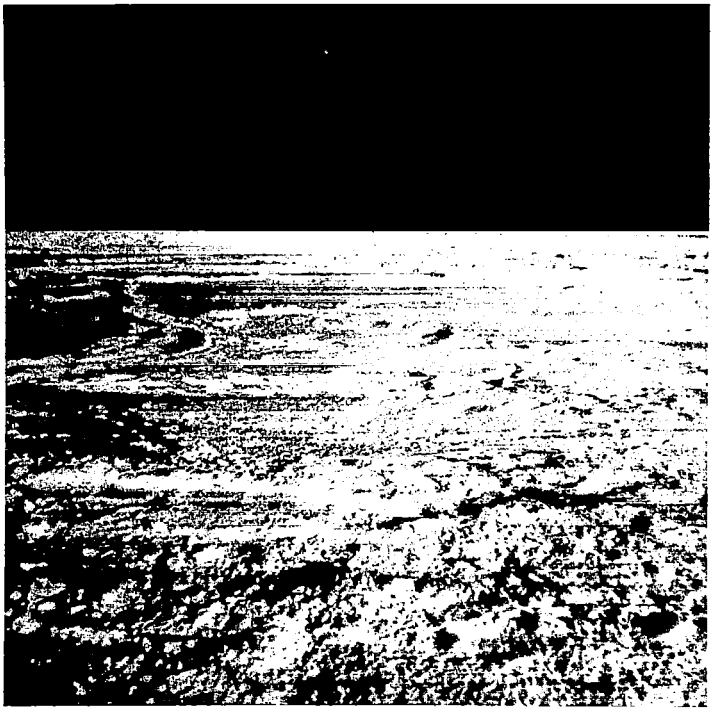

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Figure 1. Evaporator Pond, 3 January 1965

Table 4

Sulfide Concentrations in Evaporator Pond Samples

Location	Date	Sulfide, mg/ℓ
Pond	22 May 1965	16.4
Pond	6 June 1965	8.4
Pond	6 July 1965	19.5
Brine Entering Pond	22 May 1965	32.8
Brine at Source*		
I. From Area of Salt Body	6 July 1965	30.6
II. 0-6 Inches Below Surface	6 July 1965	11.2
Wadi Natrûn Lakes		222-415

* Brine Well, Owens Lake Proper

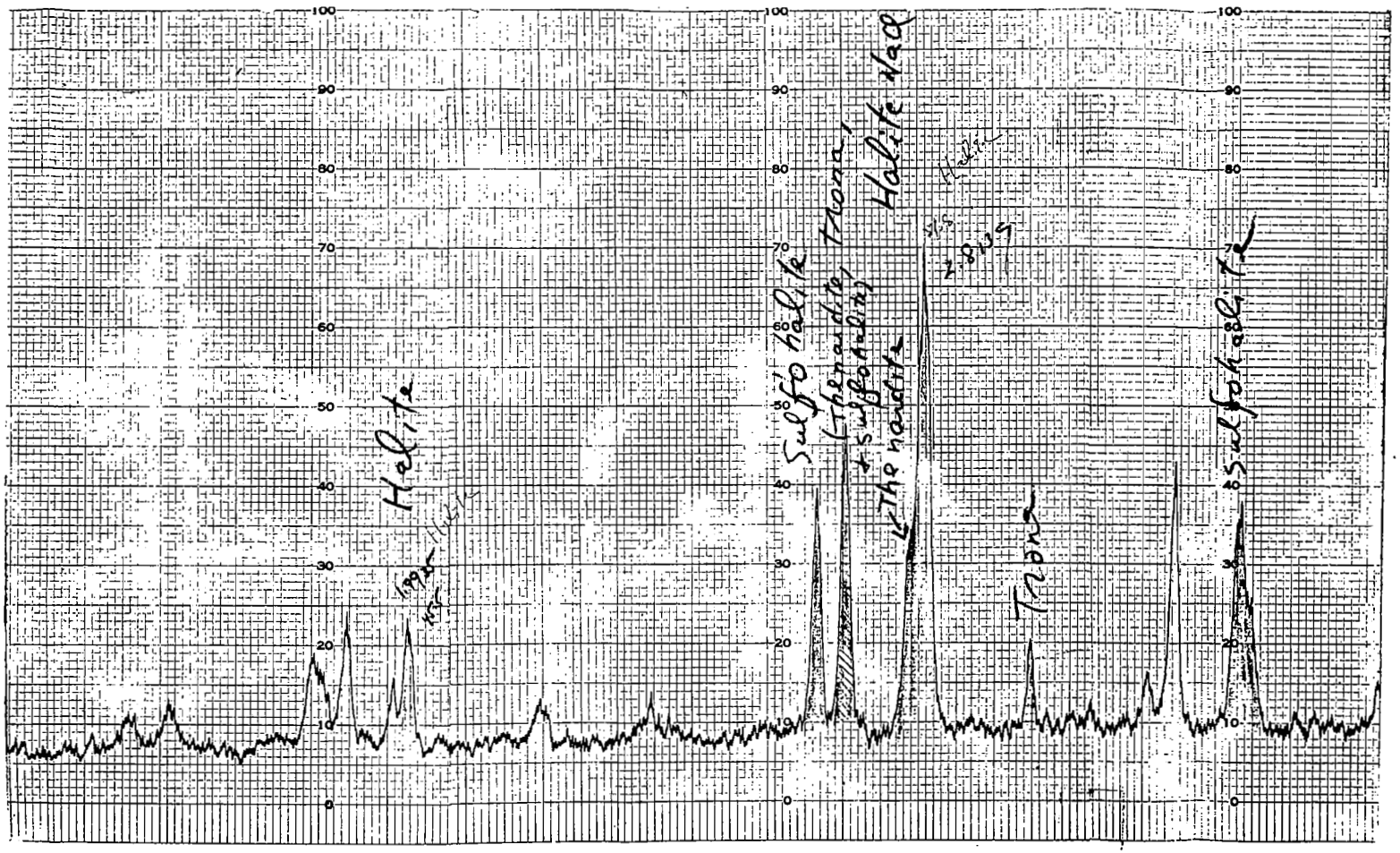

Figure 2. Solid Phase Composition by X-Ray Diffraction,
I. White Layer, 27 July 1964

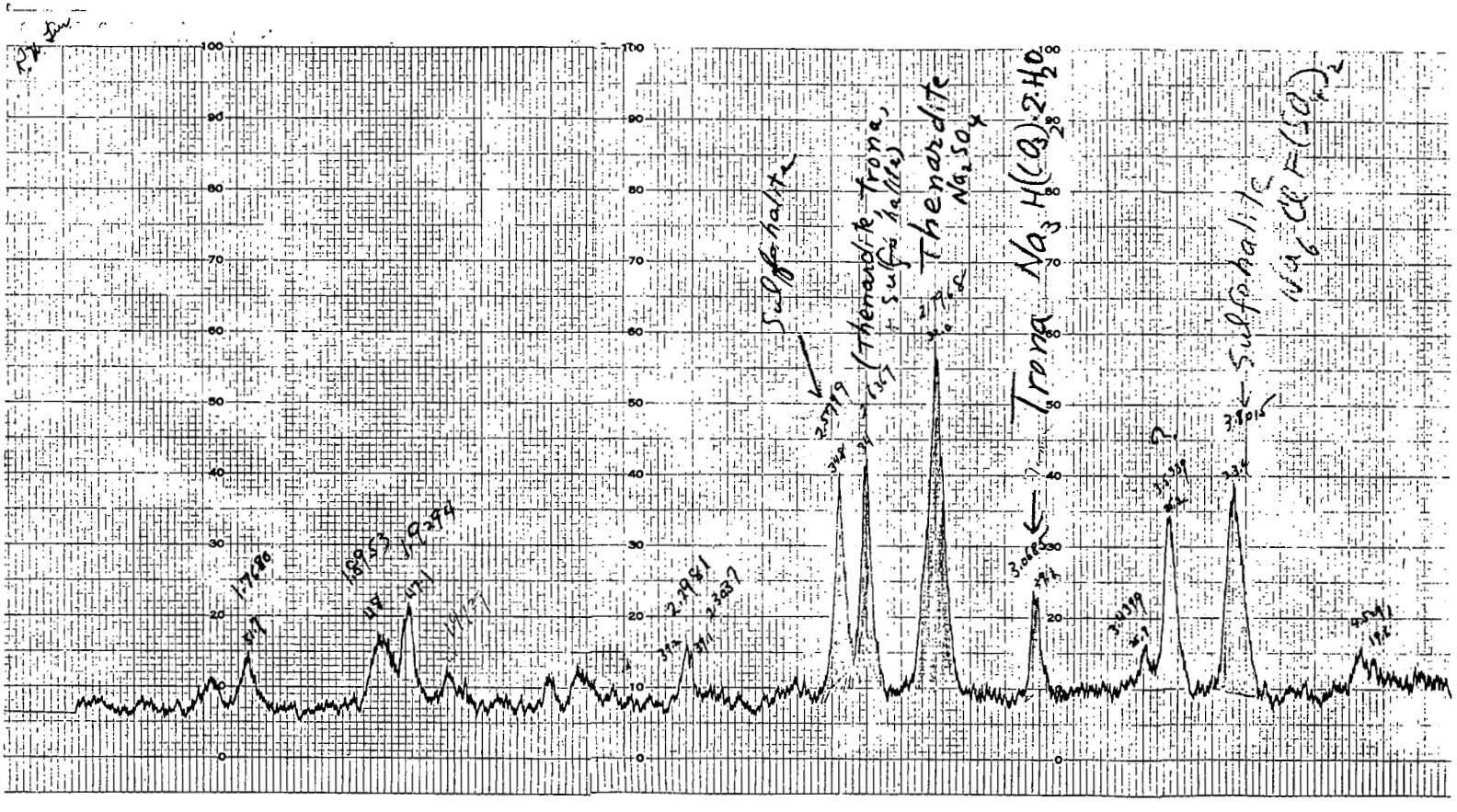

Figure 3. Solid Phase Composition by X-Ray Diffraction,
II. Purple Layer, 27 July 1964

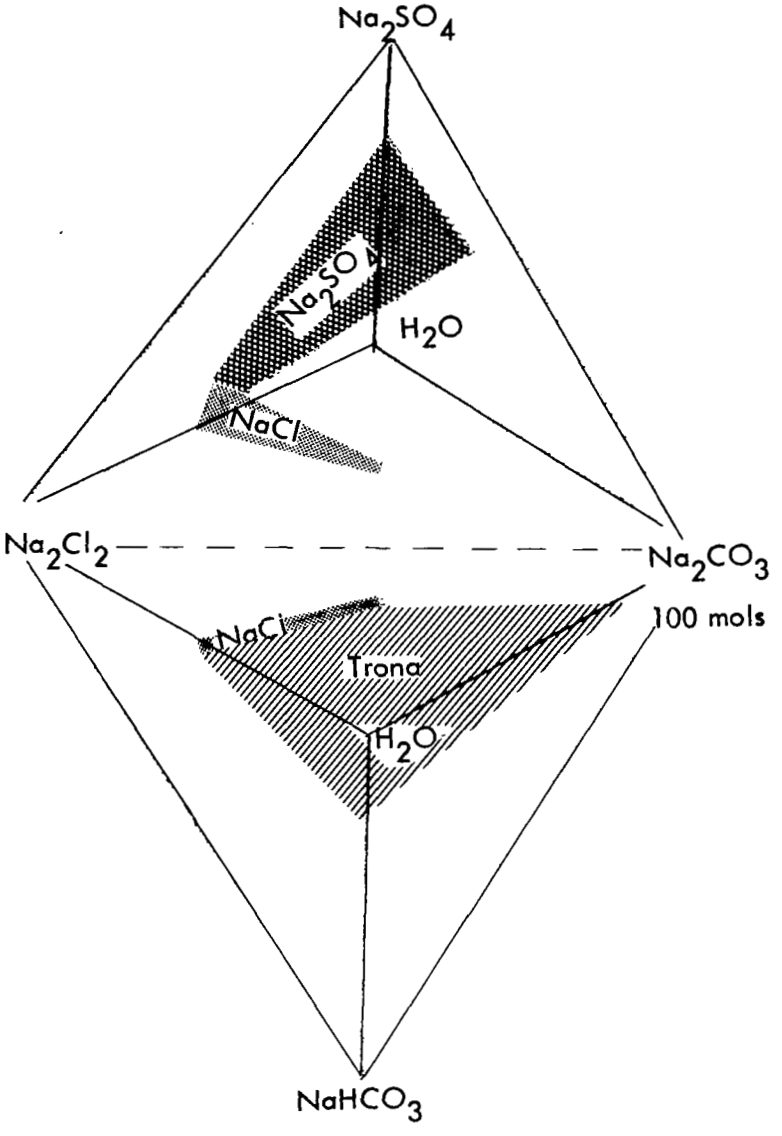

Figure 4. The Systems Sodium Sulfate - Sodium Carbonate - Sodium Chloride and Sodium Chloride - Sodium Carbonate - Sodium Bicarbonate at 35°C

the water axes, or, as they would appear from above. It is easy to imagine the structures as they would appear if the H_2O-NaCl-Na_2CO_3 faces of each were brought together, to envision a band of NaCl insolubility in a plane slightly below the resulting water apex, and to note that thenardite (Na_2SO_4) and trona will both be insoluble at certain rather limited concentrations within this "band". These concentrations must prevail in the brine when all three salts are precipitated at 35°. The bands of trona and sulfate insolubility are also three dimensional, and presumably extend from the planes illustrated to opposite faces of the tetrahedra.

Table 5 indicates the relative proportion of salts in brine from which sulfohalite, halite, thenardite, and trona precipitate at 35°C. The results are given in moles percent, a datum unrelated to the total volume of solvent.

Analyses of July, August, October, and November salt cake samples by the Megregian-Maier procedure revealed less than .05 mg fluoride per 100 mg sample. This is a very sensitive procedure; its limit is .05 mg F^-. The method is influenced by $CO_3^=$, $SO_4^=$, Cl^-, and alkalinity, but not to a significant degree with the sample sizes used. However small, the possibility of interference was important enough to warrant substantiation or denial. Therefore, a few samples should have been steam distilled prior to reanalysis by the Megregian-Maier procedure. Confirmation of the absence of fluoride would reopen the question of the identity of the mineral yielding the X-ray diffraction peaks formerly assigned to sulfohalite.

Samples of the pink "growth layer" from the lake were analyzed for sulfate, chloride, and carbonate. The results presented in Table 6 indicate that the percentage of chloride salts in the layer rises markedly in late fall and winter. The samples were merely drained of brine (when brine was present at all) and were not dried before analysis; therefore, the percentage of water or existence of salts other than carbonates was not determined. The shape of the carbonate titration curves indicated that trona was not the only carbonate present.

When interpreted by phase diagrams in References 9 and 10 and Figure 4, the brine and solid phase compositions given in Tables 3 and 6, at a pH of 10.3, are consistent with the presence of thenardite, trona, halite,

Table 6

Analyses of Evaporator Pond Salt Deposits

Sample	Chloride, as NaCl	Carbonate, as Na_2CO_3	Sulfate as Na_2SO_4	Not Accounted For
27 July	43	220	581-630	107-156
19 August	52	232	446-529	187-270
31 October	74	244	550-576	106-132
27 November	277	176	441-414	106-133
3 January	437	116	279-281	166-168

1. Values are in mg per gram sample.

2. Samples were taken from the purple salt layer, and were not dried to constant weight before analysis.

Table 5

Proportions of Salts in Brine from Which Sulfohalite, Halite, Thenardite and Trona May Precipitate at 35°C

Salt	Moles %	Molecular Weight	Grams (regardless of volume)
NaCl	60	58.5	35.1
Na_2SO_4	12	142	4.0
NaF	8	42	3.4
Na_2CO_3	12	106	12.7
$NaHCO_3$	8	84	6.7
	100		
(H_2O*	30	18	5.4)

* Water of hydration for trona

and probably thermonatrite, (Na_2CO_3-H_2O) in the solid phase at temperatures above 30°. Therefore, these salts have been used in experimentation with photosynthetic isolates. The borate indicated in Table 3 has not been included in these experiments. At temperatures between 17° and 30°C, Na_2CO_3-$10H_2O$ (natron) and Na_2CO_3-$7H_2O$ may occur. Below 17°C mirabilite might be found.

It is important to note the importance of temperature and concentration on hydration transitions of a given salt. For example, sodium sulfate in saturated solutions passes from the decahydrate (mirabilite) to the anhydrate (thenardite) at temperatures above 32°C. When saturation concentrations of NaCl are also present, the invariant point is 17.9°C. Similar considerations govern the identity of sodium carbonates in solution, although here the situation is complicated by pH and the partial pressure of CO_2.

Because of their effect on calculations of water activity, phase changes have been of prime importance in experiments on the water relations of the photosynthetic isolates. Also, anhydrate-hydrate transitions as well as solubility effects and brine withdrawal and evaporation might play an important part in the solidification of the evaporator pond during periods of declining temperature.

3.2 ISOLATION AND CHARACTERISTICS OF PHOTOSYNTHETIC HALOPHILES

3.2.1 ENRICHMENT CULTURES

Enrichment for isolation of pure cultures and preliminary determinations of salt tolerance maxima were accomplished simultaneously. This was done by performing triple factorial experiments, with inocula consisting of 0.1 gram of salt cake, and the variables being Na_2SO_4, NaCl, and either Na_2CO_3 · $NaHCO_3$ · $2H_2O$ (trona) or mixtures of $NaHCO_3$ and Na_2CO_3. The composition of the nutrient medium is indicated in Table 1. Table 7 is a representation of the experimental design for one of the experiments, and also contains data on the interval between inoculation and initiation of growth. The cultures were not harvested for determination of growth, but were retained as sources for isolation.

Table 7

Days Required for Initiation of
Growth in Enrichment Cultures

1. $NaHCO_3$: 27 gms/l; Na_2CO_3: 40 gms/l

Na_2SO_4, gm/l \ NaCl/gm/l	51	102	153	205
50	7	17	21	19*
75	6	17	21	18*
100	21*	20*	25*	20*

11. $NaHCO_3$: 41 gms/l; Na_2CO_3: 60 gms/l

Na_2SO_4, gm/l				
50	18	17	25	20*
75	11	17	25	18*
100	17*	20*	18*	21*

111. $NaHCO_3$: 54 gms/l; Na_2CO_3: 80 gms/l

Na_2SO_4, gm/l				
50	17-D*	25*	18*	17*
75	18*	20*	19*	18*
100	17-D*	21*	25*	21*

1. Inoculum: 0.1 gm salt cake (July)
2. * Denotes presence of precipitated salts
3. D indicates that growth was predominantly green flagellates

Bacteria of varying morphology (rods, vibrios, spirals) were visible in the primary enrichments, either as single motile cells moving about among salt crystals and sulfur granules, or incorporated in masses of slime. Some appeared to contain sulfur. The cultures were obviously multispeciate.

Growth of purple bacteria generally occurred throughout the factorials. Green flagellates predominated in two cultures containing low concentrations of NaCl.

Serial transfers were made from cultures containing high, low, and "medium" salt concentrations, where low consisted of 51 gm NaCl, 67 gm Na_2CO_3, and 50 gm Na_2SO_4, per liter and "high" designated saturation levels of these salts, into media of the same composition. In general, the morphology of the organisms in the cultures became less variable as the number of transfers increased. After 2 to 4 transfers, only rods similar to those recovered in agar and a few spiral forms were noted.

Bacteriochlorophyll appeared to be present in the high-salt enrichment cultures. Methanolic extracts prepared according to Reference 1 were found to have absorption maxima at 770 mμ, at 610 mμ, and at 360 mμ with a shoulder at 390 mμ. These peaks are indicative of bacteriochlorophyll.

A second enrichment experiment in salt requirements revealed unexpected interactions between NaCl, Na_2SO_4 and trona ($Na_2CO_3 \cdot NaHCO_3 \cdot 2H_2O$). The design, concentrations of salts, and total growth after 15 days are recorded in Table 8. The organisms grew well in saturated Na_2SO_4 if NaCl and trona were present in rather low concentrations, and in saturated trona if NaCl and Na_2SO_4 were present in relatively small amounts. In media saturated with NaCl or with trona and Na_2SO_4, growth 15 days after inoculation was limited to the salt cake and did not occur throughout the brines until 30 days. In the cultures containing 20 grams of trona and less than saturated NaCl the onset of growth and colloidal sulfur formation was evident within three days. At the other extreme of salt concentrations, growth was observed in solutions containing less than 1% total salts.

Highly motile spiral and rod shaped bacteria were found in droplets of brine accumulating around salt cake samples (January 3, 1965) equilibrated with

Table 8

Growth Response of Evaporator Pond Bacteria
To Sodium Chloride, Sodium Sulfate and
Trona in Enrichment Culture

1. 2 Grams Trona

	NaCl			
Na_2SO_4	0	3.5	35	Saturated
0	.450	.600	.950	0
1.43	.550	.420	1.30	0
14.3	.750	.820	1.62	0
Sat	0	0	0	0

2. 20 Grams Trona

0	1.98	1.40	2.00	*
1.43	1.30	1.50	1.20	*
14.3	1.25	1.30	1.20	*
Sat.	1.10	1.15	1.05	*

3. Saturated Trona

0	1.72	1.05	.900	*
1.43	.950	.950	.950	*
14.3	.400	1.00	1.25	*
Sat.	*	*	*	*

---

* Indicates growth on salt cake after 15 days, suspended after 30 days.

1. Readings are optical density at 780 mμ after 15 days. Values over .500 were obtained by dilution, O.D. determination, and multiplication by the dilution factor.

a high relative humidity atmosphere and incubated under low light intensity. The spirals predominated initially, but were soon replaced by rods which, however, did not resemble those found in enrichments or the purified cultures. Two days after the appearance of the bacilli the brine appeared to be essentially sterile. It should be noted that the droplets of brine were incubated in the presence of atmospheric oxygen.

No growth was noted in heterotrophic media (Tryptone-Glucose-Extract) supplemented with 15% NaCl, inoculated with samples of brine (August, 1964; June, 1965) or salt cake (3 January); and incubated in the dark. Desulfovibrio was not found upon enrichment in modified Starkey's medium. A non-photosynthetic gram-negative rod was found in one of the enrichment cultures.

3.2.2 ISOLATION OF HALOPHILIC CHROMATIUM

Several isolates have been obtained from salt deposit enrichment cultures by roux tube methods. All were of sufficiently similar morphology to be considered members of at least the same genus. Two of these gram negative isolates were examined for the presence or absence of possible contaminants. These were (1) heterotrophic bacteria, (2) sulfur oxidizing aerobes, and (3) Athiorhodaceae. In a practical sense, contaminants could persist only if capable of growth under halophilic conditions. Therefore, the media employed were appropriate modifications of the solution for culture of photosynthetic halophiles in agar (for which see Materials and Methods). The details are given in Table 9.

One of the isolates was contaminated with a facultative, heterotrophic, gram negative rod. This isolate was repurified.

The other isolate (designated H-10) contained no heterotrophic or other contaminants. In autotrophic media, the cultures appeared predominantly to contain rods and vibrios. Occasionally, forms resembling spirals could be seen, but on close observation cross walls were observed at points of inflexion. The individual "segments" were vibrio and rod shaped and did not resemble the true spirals found in enrichments or in droplets of brine accumulating around salt cake samples in high RH atmospheres.

Table 9

Media for Determination of Purity of Isolates

1. For Heterotrophs: Add 20g trona, 2g glucose, 10g tryptone, 1g yeast extract, and 15 grams agar per liter. Inoculate stabs, streak plates, incubate in light and dark.

2. For Beggiatoa: Add 20g $NaHCO_3$, 4.8g Na_2S, and 4g $Na_2S_2O_3$ per liter. Place 50 ml medium in 500 ml erlenmeyer flasks, inoculate, and incubate in the dark.

3. For Thiobacillus spp.: Add 0.1g $NaHCO_3$, 1.0g powdered sulfur and 5.0g $Na_2S_2O_3$. Treat as in 2 above.

4. For Athiorhodaceae or Thiorhodaceae growing on organic hydrogen donors: Add 5g ethyl alcohol, 1.0g malic acid, 1.5g yeast extract, 20g trona and 15g agar to 1 liter of basal solution.

5. Where autoclaving would have seriously altered the media, the agar and non sensitive constituents were sterilized by autoclaving, and sensitive components by pasteurization and/or filtration.

6. All components were added to nutrient solution also containing 60 grams NaCl and 35 grams Na_2SO_4 per liter.

In heterotrophic stabs incubated in the light and in essentially mineral media containing glutamate and malate, the usual purple anaerobic growth was noted, along with a tendency toward chain formation. Upon retransfer to autotrophic media, resumption of typical rod and vibrio morphology was noted.

No growth occurred anaerobically or aerobically in autotrophic or heterotrophic media incubated in the dark or in autotrophic media lacking sulfide and thiosulfate. Refractile inclusions occasionally appeared in a very few cells in late logarithmic or stationary phase. These may or may not have been sulfur inclusions, and were not polarly arranged. Evidence from observation of the cells under a wide variety of conditions and at various stages of growth indicate that these bacteria do not accumulate sulfur.

At the optimum water activity of .95 (liquid media) and at 32° the dimensions of the highly motile cells were 1.3x2.9 μ one day after inoculation (lag phase), .3x2.8 μ after 3 days, and 1.3x3.8 μ after 6 days (stationary phase). In saturated media dimensions were 1.00x1.33 to 1.7x4.0 with an average of 1.3x2.3 μ. The isolate was found to contain bacteriochlorophyll.

The data in Table 10 justify the choice of sulphide and thiosulfate concentrations used in liquid cultures throughout the present investigations. Apparently either $Na_2S_2O_3$ or $Na_2S \cdot 9H_2O$ may be used alone if the concentration is sufficiently high. On a molar basis sodium sulfide alone seems a better sulfur source than sodium thiosulfate alone. The marked stimulatory effect obtained by adding small amounts of sulfide (.024 to .24g) to solutions containing 2.5g $Na_2S_2O_3$ indicates that part of the function of Na_2S in promoting growth is non-specific. For example, in solutions containing 20g trona/l and 2.5g $Na_2S_2O_3$, optical densities of .52 and .17 were recorded for cultures with and without 0.24g $Na_2S \cdot 9H_2O$. There seemed to be little or no interaction with trona, although this had been indicated by a pilot experiment.

As indicated in Table 11, a marked improvement in yield was obtained by increasing the concentration of thiosulfate to 5g/liter. At this point experimentation along these lines was terminated and concentrations standardized at 5g $Na_2S_2O_3$ and 2.4g $Na_2S \cdot 9H_2O$ per liter.

Table 10

Interactions Between Sodium Sulfide, Sodium Thiosulfate, and Trona

I. 2g Trona per liter

$Na_2S_2O_3$ –	$Na_2S.9H_2O$ 0	.024	.24	2.4
0	0	0	0	0
.025	0	0	0	0
.25	0	0	0	0
2.5	.07	.03	.07	.18

II. 20g Trona per liter

$Na_2S_2O_3$ –	$Na_2S.9H_2O$ 0	.024	.24	2.4
0	0	0	0	.54
.025	0	0	0	.50
.25	0	.08	.11	.75
2.5	.17	.25	.52	.18

III. 200g Trona per liter

$Na_2S_2O_3$ –	$Na_2S.9H_2O$ 0	.024	.24	2.4
0	0	0	0	.70
.025	0	.0	.05	.55
.25	0	.0	.22	.48
2.5	.13	.68	.48	.75

1. Data are optical densities at 780 mμ after 10 days incubation. Each datum represents the mean for four cultures.

2. Medium contained 143g Na_2SO_4, 40g NaCl per liter.

Table 11

Influence of pH on Growth

	Na Carbonates, g/l		
pH	3	30	Saturated
8.4	.17	.19	.75
9.3	3.8	3.0	.18
10.3	1.5	.18	.09

Data are optical densities at 780 mμ after 10 days incubation.

Medium contained 143g Na_2SO_4, 40g NaCl, 5g $Na_2S_2O_3$, and 2.4g $Na_2S.9H_2O$. At pH 8.4, carbonate was added as $NaHCO_3$, at pH 9.3 as trona, at pH 10.3 as Na_2CO_3.

In interpreting this data the multiple possible functions of the sulfur compounds should be recognized. Hydrogen sulfide may act as an oxygen scavenger[3] (the reaction produces water and sulfur), auxiliary hydrogen donor, or as a source of the latter. Hydrogen sulfide may also affect metabolism by rendering essential elements unavailable. This situation, the extracellular catalysis of sulfide formation, plus the complex series of non-biological oxidation products of thiosulfate (and the effects of pH on these processes), renders conclusions on the actual auxiliary photosynthetic hydrogen donors in the cultures rather difficult.

The optimum pH for growth of the halophilic Chromatium appears to be approximately 9.3, as indicated by the data in Table 11. The results also showed that growth in saturated carbonate solutions was better at pH 8.4 than at 9.3 and 10.3. This result can perhaps be explained by the higher a_w of saturated solutions of $NaHCO_3$.

Results of experimentation on the possibly stimulatory effects of Pfennig[9] growth factors indicated that ascorbic acid was the factor responsible for growth acceleration. This was most likely due to the oxygen scavenging action of ascorbic acid.

Rather striking color changes were observed during growth of the organisms in agar stabs. Within two days of inoculation, orange-red growth appeared along the line of the stab. A day later a yellow band (absorption maximum 290 mμ) appeared near the agar surface, and proceeded downward toward the bottom of the tube. The yellow color was probably attributable to polysulfides[3]. In the wake of the yellow band, white turbidity appeared in the uninoculated area of the agar and particularly along the line of growth. Microscopic examination showed that this turbidity was apparently caused by sulfur present as 1-2μ globules. Concurrently, the bacterial growth assumed the typical purple hue of the Thiorhodaceae. At this point, this growth appeared to be practically encrusted with sulfur.

Growth in liquid autotrophic media was preceded by development of a yellow color (polysulfides) and precipitation of elemental sulfur. The rate of onset of this "sulfur shower" appeared to be a function of inoculum size and

an inverse function of salt concentration. When 1 x 10^7, 2 x 10^6, and 5 x 10^5 washed cells were inoculated into 10 ml volumes of "low salt" brine, the yellow polysulfide color appeared after 6.3 (σ = 1.8), 8.3 (σ = 1.1) and 10.2 (σ = 1.7) days. This low salt brine contained 51, 50 and 57 grams of NaCl, Na_2SO_4, and sodium carbonates per liter. The appearance of polysulfides was noted 14 (σ = 1.4) and 17 (σ = 0.54) days after inoculation of 2.7 x 10^7 and 5.4 x 10^6 bacteria into high salt media, which contained 220, 115 and 149 grams of the NaCl, Na_2SO_4, and trona. (The inocula were taken from cultures (fourth serial transfers) of identical salt concentration.) These results parallel observations recorded by Van Niel[3].

At this point it is apparent that the halophilic isolate belongs to the family Thiorhodaceae, genus Chromatium. However, species assignment is unclear. The organisms do not accumulate sulfur, therefore do not resemble the Chromatium vinosum isolated from Lake Beloe and discussed in Reference 11. Perhaps the most suitable assignment is to Van Niel's[3,12] group of small Chromatium species which promote the formation of sulfur in the growth medium.

Koch's postulates have been fulfilled. The Chromatium isolate was found capable of growth in pasteurized natural brine and salt cake, supplemented with sulfide and thiosulfate. Uninoculated controls revealed no growth.

Figure 5 shows the halophilic Chromatium resting on a salt crystal, probably sodium sulfate.

3.3 LIMITATION OF GROWTH

Results cited to this point have substantiated the ability of photosynthetic halophiles to grow in solutions saturated with Na_2SO_4, NaCl, and trona ($NaHCO_3 \cdot Na_2CO_3 \cdot 2H_2O$) at 32°C. However, long lag phases and limited total growth have been the rule under these conditions. The situation is reminiscent of the experience of Christian and Scott[13] with Salmonellae, who found that reduction of a_w below .199 led to an increase in the lag phase and to a reduction in the total yield of cells. The evaporator pond bacteria thus may be in water limiting environments in the natural environment and in saturated laboratory cultures.

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Figure 5. Chromatium on a Salt Crystal

Before water limitation can be proven, three conditions must be met. The first two are cited in Reference[14] as follows: "The first condition is that a_w must be controlled at a known level, and must be constant in space and time for the duration of the experiment."

"The second general condition is that the relation between the response and a_w should be measured in more than one medium . . . to ensure that the response in a function of a_w and not of the concentration of a particular solute or solutes". For this reason, it is advisable to design experiments in which salt preferences and interactions are examined at concentrations of mixed or individual salts predetermined to result in the same water activity of the solution.

The third condition is that no other factors than water be limiting. From a classical Blackman's law point of view, limitation by other factors should be of little concern if consistently longer lag phases and lower total growth are observed in solutions of progressively decreasing water activity. Practically speaking, though, the fact of non-limitation by other factors was not known, and needed to be established, or at least explored by experiment.

3.3.1 GROWTH AND WATER ACTIVITY IN SATURATED SOLUTIONS

Water activities of solutions of individual salts may be calculated from the following equation[14], provided osmotic coefficients are known.

$$a_w = e^{-\frac{\nu m \phi}{55.51}} \qquad (1)$$

In this equation, ν is the number of ions generated by the solute; m its molality; and ϕ, the osmotic coefficient.

In concentrated solutions of more than one salt, the contributions of each to the total lowering of a_w may be taken additively as values of $1 - a_w$, provided the second approximation of Robinson and Stokes[15] holds for colligative properties other than lowering of vapor pressure.

Osmotic coefficients for sodium chloride and sodium sulfate are available in the literature[16], therefore, the contribution of each salt to a_w

may be calculated from the appropriate value of ϕ for the molalities indicated in Table 12. The data in the table were obtained by analysis of brines saturated with NaCl, Na_2SO_4, and $NaHCO_3 \cdot Na_2CO_3$ at 25°C and pH 9.5.

For a 4.5 m solution of NaCl, a_w is 0.827. The value of $1-a_w$ for a 0.55 m solution of Na_2SO_4 is 0.022. The total water activity is 0.805 for mean values of m for the two salts.

The situation with the carbonates was somewhat more complex in that values for ϕ could not be found in the literature. Activity coefficients, γ, for bicarbonate and carbonate were available in References 17 and 18, and were converted to osmotic coefficients by graphical integration of the equation[16],

$$\phi = 1 + \frac{1}{m}\int_0^m m d\ln \gamma \qquad (2)$$

between limits of m = o and m = m. The decision to integrate between these limits, rather than the much more difficult $\gamma = 0$ to $\gamma = m$, was justified by integration between m = o and m = m for values of γ for Na_2SO_4, and comparison of values of ϕ so derived with literature values. The calculated osmotic coefficients for $NaHCO_3$ and Na_2CO_3 are given in Table 13. It will be noted that the data for bicarbonate are significant only to two places.

From the data in Reference 17, the fractions of sodium ion (g ions/l) in carbonate-bicarbonate solutions at pH 9.5 are .63 and .37, respectively. Accordingly, results of titration expressed as 66.7 g Na_2CO_3 are equivalent to 42 g Na_2CO_3 and 57 g $NaHCO_3$. On a molal basis, the brines were thus 0.47 m in Na_2CO_3 and .78 m in $NaHCO_3$. Calculated values of $1 - a_w$ were .013 and .036 (again for mean values of m, and with $\nu = 3$ for both salts).

The water activity of the saturated brine was thus 0.805 - 0.013 - 0.036, or 0.756. Since the data for bicarbonate are valid to two significant figures, this value becomes 0.76.

The total a_w of the brine was also calculated for pH 9.3, where the fraction of g/ions Na/l as carbonate and bicarbonate was deduced to be 0.5, from Reference 17. At pH 9.3, the total a_w was 0.74, again using values of ν = 3 for bicarbonate. This datum for a_w is approximately 0.02 below that for a 6 m

Table 12

Analyses of Saturated Brine

Salt	Grams per Liter		Molality*	
	X	σ	pH 9.3	pH 9.5
NaCl	224	4.14	4.50	4.5
Na_2SO_4	73.1	6.72	.60	.58
Carbonate as Na_2CO_3	66.7	0.42	.38	.47
$NaHCO_3$			1.14	.78

* Calculations of H_2O concentration include 14 grams total of minor constituents. Specific gravity of brine, 1.260. Weight of 10 ml brine, 12.66 50 g.

Table 13

Calculated Osmotic Coefficients for Sodium Carbonate and Sodium Bicarbonate

m	$NaHCO_3$ ϕ	m	Na_2CO_3 ϕ
0.1	.88	0.1	.820
0.2	.87	0.2	.770
0.3	.87	0.4	.765
0.4	.87	0.6	.751
0.6	.87	0.8	.744
0.8	.86	1.0	.741
1.0*	.88	1.2	.735
1.2*	.87	1.4	.723
1.4*	.89	1.6*	.722

* Extrapolated values of m and γ .

saturated solution of NaCl alone, which lends doubt to the validity of the former on the basis of actual physical meaning. When $\nu = 2$ is used for bircarbonate at pH 9.3, the total a_w of the solution becomes 0.76, which is perhaps more in line with the concept of solubility in a solution of more than one saturated component as a function of competition for water.

3.3.2 INFLUENCE OF THE SOLUTE ON WATER LIMITATION OF GROWTH

According to Scott[14], "The second general condition (for proof of water limitation of growth) is that the relation between the response and a_w should be measured in more than one medium . . . to ensure that the response is a function of a_w and not of the concentration of a particular solute or solutes." An experiment with the halophilic Chromatium has been performed to test compliance or noncompliance with this condition.

Briefly, a basal solution containing increments of Na_2SO_4, NaCl, and $NaHCO_3$ (pH 9.5) was prepared so that each salt contributed an increment of $1-a_w$ of .01 to the solution. The calculated a_w of the basal solution was thus .97. Increments of each salts were then added separately to 200 ml quantities of basal solution to reduce a_w by the values indicated in Table 14. Fifty ml of the resulting solutions were then added to each of four flasks, inoculated, and incubated at 32°C. After 17 days analyses for chloride, carbonate, sulfate, optical density at 780 and 525 mμ, Eh and pH were performed. Results of these analyses are given in Tables 14, 15, 16, and 17. Computations of a_w from analytical data agreed rather well with a_w intended.

Figures 6a, 6b, and 6c illustrate this experiment as it appeared on the third, fifth, and seventeenth days of incubation. Intended water activity increases from .78 at the left of each photograph to .97 at the extreme right, as indicated by the values of $1-a_w$ lettered on each flask. Growth can be seen to proceed in the reverse direction, with an optimum near .95. At this water activity, growth was evident after 4 days. The yellow coloration proceeding the "sulfur shower", and the "sulfur shower" which appears before growth becomes obvious are visible in the flasks marked SO_4-1 in Figure 6a, and in flasks SO_4-04, Figures 6a and 6c. Preference for chloride may be noted in Figure 6c. Again, an increase of Eh to positive values accompanied growth.

Table 14

Growth After 17 Days in Solutions of Decreasing Water Activity, I.
Response to a_w in More Than One Medium

Optical Density at 780 m

Water Activity (Intended)	NaCl Series		Na_2SO_4 Series		Carbonate Series	
	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ
.78	.09	.08	-		-	
.84	.07	.06	.03	.01	-	
.87	.04	.01	.05	.02	-	
.90	2.8	.2	.05	.02	2.0	1.0
.93	4.7	.74	1.6	1.06	4.7	1.1
.95	5.2	.12	6.0	.4	5.7	.6

OD of Growth in Basal Solutions: 5.7 (σ = .33) at a_w = .97.

Basal Solution: a_w = .97, by addition of NaCl, Na_2SO_4, Na_2CO_3-$NaHCO_3$, (pH 9.5) each at a concentration of 1-a_w equalling 0.01.

Table 15

Growth After 17 Days in Solutions of Decreasing Water Activity, II
Detailed Results for Solutions of NaCl

Optical Density

Water Activity		525 mμ		780 mμ		pH		Eh	
Intended	By Analysis	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ
.78	.78	0	-	.09	.08	8.71	.04	-.20	.02
.84	.84	.01	.005	.07	.05	8.88	.01	-.19	.02
.87	.87	0	.003	.04	.01	8.97	.03	-.18	.01
.90	.90	1.37	.029	2.77	.17	9.08	.01	+.261	.03
.93	.94	2.18	.090	4.54	.74	9.18	.02	+.13	.03
.95	.96	2.10	.036	5.16	.12	9.30	.01	+.18	.02
.97*	.96	2.33	.087	5.70	.33	9.38	.01	+.13	.01

*Basal Solution

Table 16

Growth After 17 Days in Solutions of Decreasing Water Activity, III
Detailed Results for Solutions of Sodium Sulfate
and of Sodium Carbonates

Water Activity		Optical Density							
		$\bar{x}$ 525 mμ	σ	$\bar{x}$ 780 mμ	σ	$\bar{x}$ pH	σ	$\bar{x}$ Eh	σ
Intended	By Analysis								
I. Sodium Sulfate									
.84	.86	.01	.003	.03	.014	9.08	.03	-.19	.03
.87	.88	.05	.002	.05	.014	9.08	.08	-.19	.01
.90	.88	.01	.005	.05	.021	9.13	.06	-.19	.02
.93	.94	.99	.52	1.58	1.06	9.18	.03	+.02	.13
.95	.95	2.42	.20	5.97	3.66	9.30	.01	+2.31	.05
.97*	.96	2.33	.09	5.70	.33	9.38	.01	+.13	.01
II. Sodium Carbonates									
.90	.90	1.25	.32	1.96	.31	9.60	.01	+.19	.01
.93	.93	2.56	.02	4.71	.31	9.46	.01	+.19	.09
.95	.95	2.79	.35	5.71	.64	9.40	.01	+.18	.02
.97*	.96	2.33	.09	5.70	.33	9.38	.01	+.13	.01

* Basal Solution

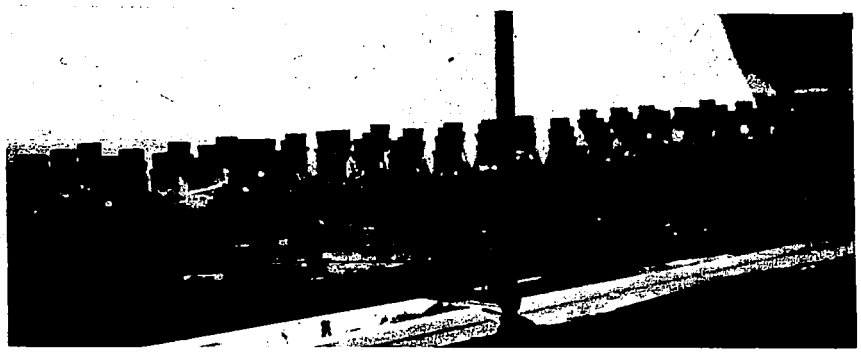
Table 17

Growth After 17 Days in Solutions of Decreasing Water Activity, IV, Analyses for Sodium Chloride, Sodium Sulfate, and Sodium Carbonate Series

Water Activity, Intended	NaCl Series Added*	NaCl Series Found	aw	Na_2SO_4 Series Added	Na_2SO_4 Series Found	aw	$NaHCO_3$ Added	$NaHCO_3$ Found	Carbonate Series Na_2CO_3 Added	Carbonate Series Na_2CO_3 Found	aw (both salts)
.78	4.92	5.08	0.78	-			-		-		
.84	3.48	3.72	0.84	3.66	3.43	0.86	-		-		
.87	2.80	3.04	0.87	2.96	2.90	0.88	-		-		
.90	2.02	2.24	0.90	2.78	2.75	0.88	0.91	0.85	1.18	1.10	0.90
.93	1.20	1.19	0.94	1.18	1.15	0.94	0.60	0.65	0.54	0.49	0.93
.95	0.61	.62	0.96	0.60	0.61	0.95	0.35	0.38	0.21	0.17	0.95
.97**	0.30	.39	-	0.30	0.30	-	0.19	.184	0.09	0.07	-

* Values in Moles/1000 grams H_2O

** Basal Solution, total aw (analyzed), 0.96



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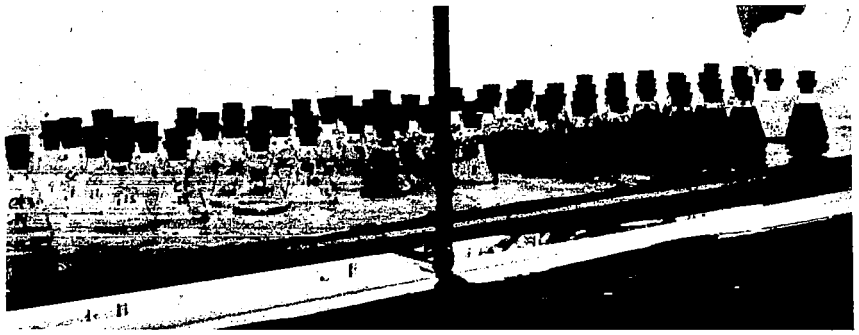
3 Days

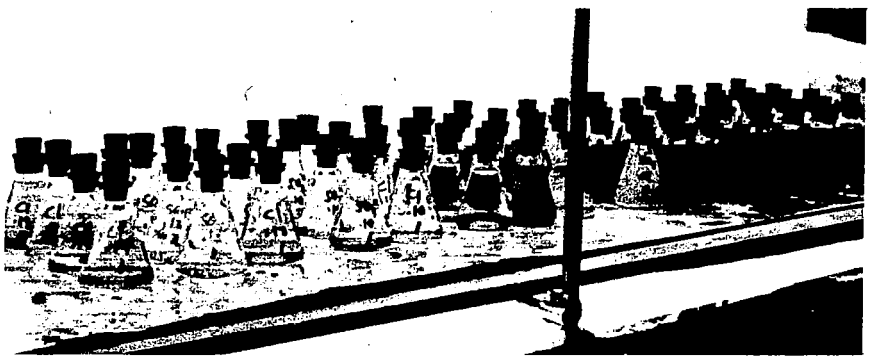
Figure 6a. Growth Response to Increased Concentrations of Individual Salts ($NaCl$, Na_2SO_4, and $NaHCO_3$-Na_2CO_3).



SGC/735

5 Days

Figure 6b. Growth Response to Increased Concentrations of Individual Salts ($NaCl$, Na_2SO_4, and $NaHCO_3$-Na_2CO_3).



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17 Days

Figure 6c. Growth Response to Increased Concentrations of Individual Salts ($NaCl$, Na_2SO_4, and $NaHCO_3$-Na_2CO_3).

The data for NaCl, Na_2SO_4 and $NaHCO_3$-Na_2CO_3 concentrations are given in Table 17, along with computed actual activities of water. When the results for growth at 17 days (Tables 14, 15, 16) are compared on the basis of actual a_w of the solutions, it seems apparent that the response may be a function of the concentration of the particular solute in grams per 1000 grams H_2O and not simply a function of a_w. Sodium chloride and sodium carbonates are probably equally tolerated, but both certainly seem preferable to sodium sulfate. Therefore, Scott's second condition is met for sodium carbonates and sodium chloride, but not for sodium sulfate. Admittedly, the data requires a more thorough analysis both in terms of significance and of the physical situation in concentrated solutions. The inoculation procedure for this experiment afforded an opportunity to determine the relative density of the cells. The inoculum consisted of equal numbers of cells from agar stabs and from liquid cultures containing saturation concentrations of NaCl, Na_2SO_4, and trona. During inoculation of the chloride series, it was noted that the organisms from the saturated culture sank in all but the a_w .78 media. The inoculum from the stabs floated in all but a_w .95 and .97 solutions. These observations, though only very tentative, indicate that the cells were in density equilibrium with the medium in which they were cultured. An exception to this conclusion was observed: the organisms from the saturated culture tended to float when added to carbonate media of a_w .90 and .93.

3.3.3 CULTURES CONTAINING A SOLID PHASE

The water activity approach has been employed in studies of growth in biphasic systems. Table 18 is illustrative. This table contains data from Reference 9 on the effect of variation in concentrations of NaCl and Na_2SO_4 in the liquid phase upon the solubility of each salt, and on the nature of the solid phase at 25°C. Points worthy of note are (1) the phase transition from thenardite (Na_2SO_4) to mirabilite ($Na_2SO_4 \cdot 10H_2O$) at brine concentrations of 13.6% NaCl and 14.76% Na_2SO_4 at 25°C, and (2) the inverse relationship of a_w to chloride concentration, despite increasing solubility of Na_2SO_4. For example, when the solid phase consists of NaCl and Na_2SO_4, liquid phase a_w is .765. With no NaCl present, the solid phase is mirabilite, and the brine has an a_w of .935. The transition

Table 18

Solubility and Water Activity Data for the System
$NaCl - Na_2SO_4$

	NaCl			Na_2SO_4			
Solid Phase	%	m	a_w	%	m	$1-a_w$	Total a_w
$Na_2SO_4 \cdot 10H_2O$	0	-	-	22.11	1.94	.065	.935
"	2.27			20.44			
"	4.42			18.59			
"	6.35	1.4	.953	16.23	1.44	.048	.905
"	9.44			14.55			
"	11.61	2.5	.913	14.42	1.34	.045	.868
$Na_2SO_4 \cdot 10H_2O$ + Na_2SO_4	13.60			14.76			
Na_2SO_4	16.57	3.9	.859	11.62	1.1	.032	.824
"	19.15	4.6	.825	9.11	.87	.029	.796
"	21.92	5.3	.794	7.04	.69	.0235	.770
Na_2SO_4 + NaCl	22.96	5.4	.788	6.86	.67	.023	.765
NaCl	23.92			4.72			
"	24.91			2.44			
"	26.26			0.0			

Phase and solubility data from International Critical Tables, molality and a_w by calculation.

point for Na_2SO_4 to $Na_2SO_4 \cdot 10H_2O$ in solutions not containing other salts in 32.5°. When NaCl is present in saturation concentrations, the anhydrate-hydrate transition temperature is 17.9°.

Artificial brines in equilibrium with the appropriate salts were prepared as indicated in Table 19, and inoculated with halophilic Chromatium. Salts were added as supplements to a basal medium containing sufficient NaCl, Na_2CO_3 - $NaHCO_3$, and Na_2SO_4 to yield an a_w of .97 at pH 9.5. As would be expected, signs of growth at 25°C were first noted in flasks containing mirabilite but with no chloride present except that in the basal medium. Temperature was maintained at 25°C to maintain concentrations equal to those in Table 18, and to avoid phase transition to thenardite (Na_2SO_4).

At 25°, significant growth will occur within 32 days in the presence of solid mirabilite at sodium chloride concentrations of 1.4 m or less and if the total a_w of the solution is not less than .905. This conclusion is justified by the data in Table 19, and is probably valid for temperatures below 25°C. The data thoroughly support the advisability of planning solid-liquid phase experiments on a water activity basis. Also, data in previous reports indicate that the conditions permitting growth in this experiment also exist at certain locations and times in the evaporator pond.

During the course of this experiment, the temperature inadvertently rose to 32°C for approximately 18 hours. Much of the mirabilite disolved, but recrystallized upon readjustment of temperature to 25°C. During recrystallization, clumps of organisms were trapped within the crystals.

This observation was confirmed by repetition. Several cultures containing a mirabilite solid phase at a_w .935 were inoculated and maintained until growth of approximately 10^9 cells per ml was observed. At this point the culture temperature was increased to 32°C overnight, upon which most of the solid phase dissolved. The cultures were then removed to a 19° incubator to initiate crystallization, and finally placed at 4°C overnight. The supernatant brine was decanted, and the large masses of purple crystals drained of excess brine and stored in a dessicator.

Table 19

Growth After 32 Days at 25°C in Cultures Containing Mirabilite and Thenardite Solid Phases

		Optical Density (780 mμ)		Cells x 10^6	
a_w	Solid Phase	$\bar{x}$	σ	$\bar{x}$	σ
.970*	None	3.65	0.09	1210	127
.935	Mirabilite	1.42	0.23	328	62.4
.905	Mirabilite	0.51	0.24	39.0	7.0
.868	Mirabilite	0.02	0.002	3.26	1.7
.824	Thenardite	0.02	0.006	No Growth	No Growth
.796	Thenardite	0.01	0.006	No Growth	No Growth
.770	Thenardite	0.02	0.02	No Growth	No Growth
.765	Thenardite Halite	0.01	0.005	No Growth	No Growth

* Basal Solution

Cells per ml at 0 days: 3.5×10^6

The organisms can apparently not only survive entrapment in crystals, but, additionally, immersion in liquid nitrogen. Some of the crystals prepared, as indicated above, were immersed in three separate sterile solutions containing mirabilite at a_w .935, then in mirabilite - .01% mercuric nitrate for 10 minutes, followed by brief immersion in sterile distilled water and sterile a_w .935 mirabilite. Part of the crystals so treated were cultured in liquid media at a_w .95. Part were immersed in liquid nitrogen for 10 minutes and cultured. Growth occurred in both instances.

The remainder of the crystals were equilibrated with nearly a 100% RH atmosphere from cotton plugs moistened in .001 m $Na_2S \cdot 9H_2O$ which were inserted in test tubes containing a crystal, sealed with a rubber stopper, and incubated at 32°C. The organisms liberated by solution of the crystals floated at the top of the liquid phase.

No organisms could be definitely identified within crystals by microscopic examination. It should be noted that corotenoids have been found within crystals of Hanksite ($9Na_2SO_4 \cdot 2Na_2CO_3 \cdot KCl$) and borate from Searles Lake cores[10].

At 32°C, the halophilic Chromatium appeared to prefer NaCl and Na_2SO_4 but not $NaHCO_3 \cdot Na_2CO_3 \cdot 2H_2O$ (trona) as solid phases for growth, although observation of growth in trona in isolated cases indicates that this conclusion suffers the ambiguity of most generalizations. At least, increased growth was not noted at interfaces between immediately contiguous salt layers. Heavy growth between partially separated layers has been occasionally noted in previous experiments. The experimental plan and results which generated these conclusions are given in Table 20. It should be noted that this was a range finding experiment not designed from water activity considerations.

However, the organism apparently does have a tendency to grow or accumulate on surfaces, as indicated by the data for cells per ml in supernatants and supernatants plus wall growth in Table 21. Also, the lag phase in concentrated brines containing boiled and dried glass wool was shorter than in brines without the added surface.

Table 20

Growth of Chromatium on Solid Phases After 32 Days

Arrangement of Phases		Growth
I.	Trona	-
	Thenardite	+
	Halite	+
II.	Trona	-
	Halite	+
	Thenardite	+
III.	Halite	+
	Trona	-
	Thenardite	+
IV.	Halite	+ +
	Thenardite	+ and +
	Trona	- +
V.	Thenardite	- +
	Trona	+ and +
	Halite	+ +
VI.	Thenardite	+ +
	Halite	+ and +
	Trona	- +
VII.	Asbestos only	+ in 16 days

Table 21

Growth Data for Halophilic Chromatium in Saturated Brines

	Light Intensity, (Foot Candles)	En	Optical Density (780 mμ)	Cell Counts, x 10^6	
				Supernatants	Supernatant Plus Wall Growth
I.	3 Days				
	Dark	-.255	0	7.6	-
	55	-.265	0	7.6	-
	792	-.247	0	6.7	-
	2450	-.262	0	4.0	-
	2450*	-.242	0	9.2	-
II.	6 Days				
	Dark	-.252	.01	4.5	5.0
	55	-.259	.02	6.0	6.2
	792	-.257	.02	10.0	12.0
	2450	-.232	.01	6.2	-
	2450*	-.252	-	6.5	-
III.	10 Days				
	Dark	-.232	.04	5.4	6.2
	55	-.232	.03	9.0	-
	792	-.224	.04	8.5	8.8
	2450	-.224	.05	7.2	8.0
	2450*	-.244	.10	27.0	-
IV.	19 Days				
	Dark	-.222	.04	-	4.4
	55	-.222	.05	-	4.6
	792	-.262	.09	-	18.0
	2450	-.222	.05	-	16.0
	2450*	-242	.07	-	11.0
	Uninoculated	-.252	-	-	-
V.	22 Days			σ	σ
	Dark	-.232	.09	6.8 1.3	11 2.7
	55	-.207	.08	26 5.6	14 3.9
	792	-.242	.07	16 3.4	24 2.7
	2450	-.237	.12	19 5.8	24 0.8
	2450*	-.222	.09	15 2.3	20 4.7
	Uninoculated	-.252	-	- -	- -

Table 21 (Continued)

Growth Data for Halophilic Chromatium in Saturated Brines

Light Intensity (Foot Candles)	Eh	Optical Density (780 mμ)	Color	Cell Counts x 10^6			
				Supernatants		Supernatant Plus Wall Growth	
					σ		σ
VI. 25 Days							
2450*	-.252	.850		32	4.6	43	4.4
VII. 28 Days							
Dark	-.252	.045		7	2.7	6.8	0.8
55	-.242	.055		7.7	2.2	7.5	1.8
762	-.257	.120		18	4.6	28	1.9
2450	-.262	.255		24	13.4	42	3.6
Not Inoculated	-.232	-					
VIII. 48 Days							
792 1.	-.052	.64	Pink	75	17	132	19
2.	-.252	.85	Yellow	50	5.8	57	13.4
2450	-.247	1.20	Yellow	31	7.2	31	7.2
2450*	-.242	1.0	Yellow	15	8.2	8.2	3.9
IX. 103 Days							
0	-.202	.051	Clear	.85	.5	2.8	2.1
55	-.212	.085	Clear	18.3	6.0	7.3	3.7
792	+.088	.66	Pink	78.6	10.8	69.6	12.3
2450	-.222	1.38	Yellow	47.2	7.1	57.2	5.7
2450*	+.208	1.18	Pink	101.0	14.1	113.0	8.8
Not Inoculated	-.212	-		-	-	-	-

* Pyrogallate in small test tubes within each flask.

3.3.4 GROWTH CURVES AT A_w .76 AND .95

In accordance with the requirements of the third condition for establishment of water limitation, a study of light limitation of growth in concentrated brines has been performed as an adjunct to determination of growth rates at a_w .76. The data in Table 21 indicate that illumination from fluorescent lamps becomes limiting for growth at 32°C at a point between 55 and 792 foot candles. Therefore, light need not be considered as a limiting factor under the experimental conditions usually employed (2450 fc). After a lag phase of 10 days, growth apparently occurred until 40 to 103 days after initiation of the experiment. The highest concentration of cells found was 1.13×10^8 per ml after 103 days at 2450 foot candles; 9×10^6 cells/ml were recorded 3 days after inoculation. It seems significant that no pink coloration was noted until late in the growth cycle, and that appearance of obvious photosynthetic pigmentation was correlated with an increase of Eh to positive values. This increase in Eh was probably correlated with disappearance of sulfide from the brines. No growth or increase in Eh was observed in the dark. The results of this experiment and observations on pigment changes agar stabs containing lower salt concentrations suggest that photosynthetic pigment formation and growth in the light may be inhibited by oxygen in the manner of Rhodospirillum molischianum[21].

When plotted, the data revealed deficiencies in the experimental plan. Actually each point represents data from a single culture sacrificed at the time of analysis. This procedure resulted in large deviations of certain points from the expected curves. Also, insufficient cultures were provided for detailed description of growth during the 30 to 103 day interval. Standard deviations were not computed for results taken during the first 17 days.

3.3.4 GROWTH CURVES AT A_W .76 AND .95

Increasing randomness of results with increasing salt concentrations is illustrated in Figures 7 and 8, in which the circles represent standard deviations plotted on a linear scale. These figures are growth curves at a_w .76 and .95. The points are means from Tables 21, 22, and 23 (cell counts at 2450 foot candles) plotted on semi-log paper. During initial stages of growth, accumulation of sulfur in the medium prevented accurate enumeration, therefore curves through the means were plotted only for data taken later than 48 hours after inoculation. Diauxie indicated by these curves may also be an artifact, or may reflect an actual revision of metabolism. The composite curve in Figure 8 and the curves in Figure 7 represent best estimates.

Generation times computed from these data are 78 and 13 hours for growth at a_w .76 and .95.

One of the a_w .76 cultures was used in an experiment on the effect of dilution on the cells. The organisms retained their mobility and did not lyse upon being placed in distilled water.

3.4 THEORETICAL APPROACH TO WATER LIMITATION

The experimental approach to determination of water limitation is valuable in that concrete results are obtained. However, experimentation should be accompanied by a theoretical approach so that specific results might be analyzed within a more general framework. For example, it is apparent that the energy required to move water into a cell is an inverse function of water activity, and that at some point the energy required to absorb water should exceed the energy available for this purpose. If photosynthesis proceeds at a finite rate (and even though this process may produce water) growth rates must decrease as water for synthesis and increase in cell volume becomes more and more expensive.

The comments of Dr. Paul Blatz (California Institute of Technology) on a theoretical approach to the problem are quoted in Appendix A.

Note: Results forwarded as this report was being prepared indicate that organisms trapped in mirability crystals can not only withstand liquid nitrogen temperatures, but subsequent dessication under vacuum.

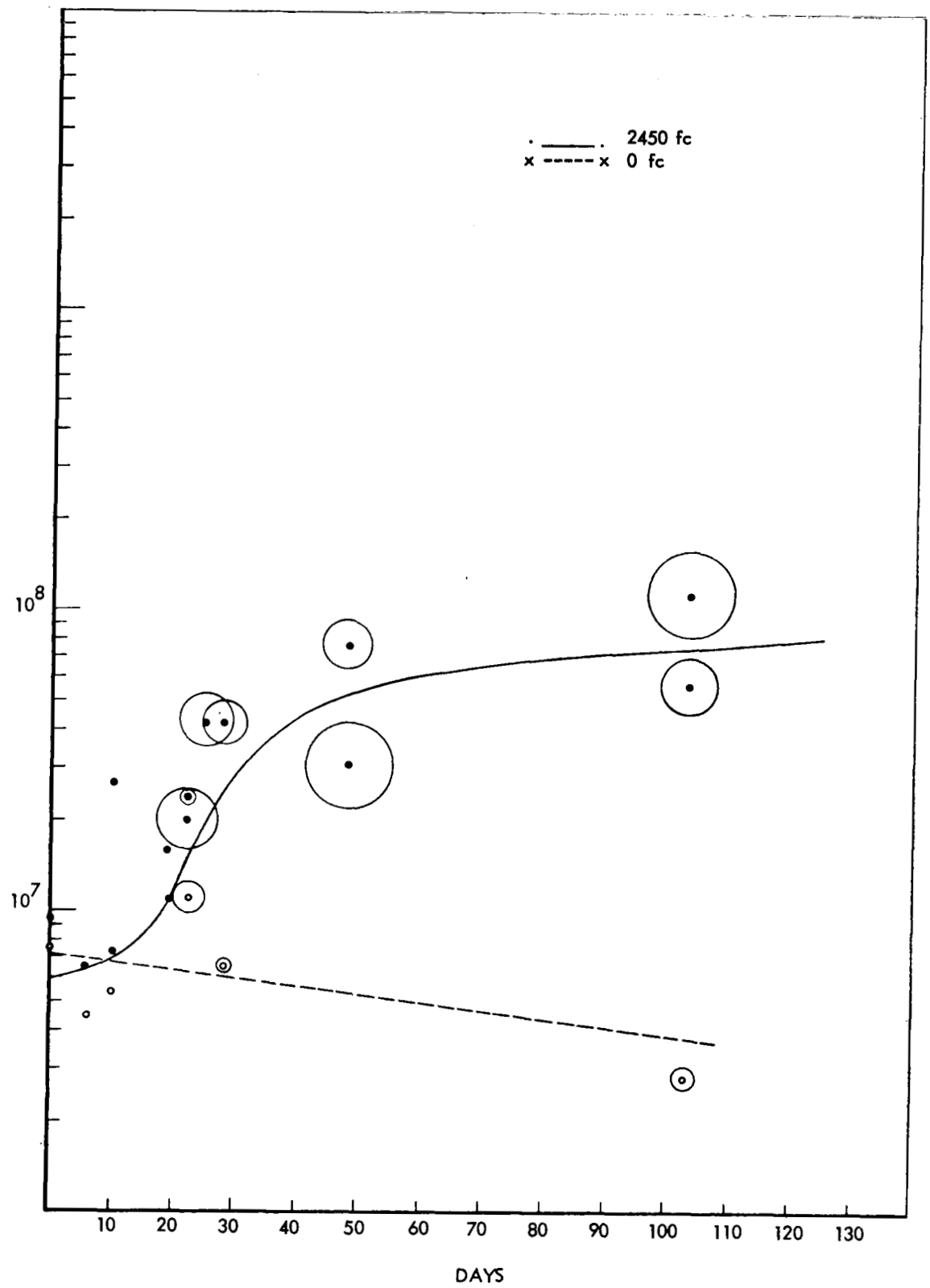

Figure 7. Growth at a_w .76

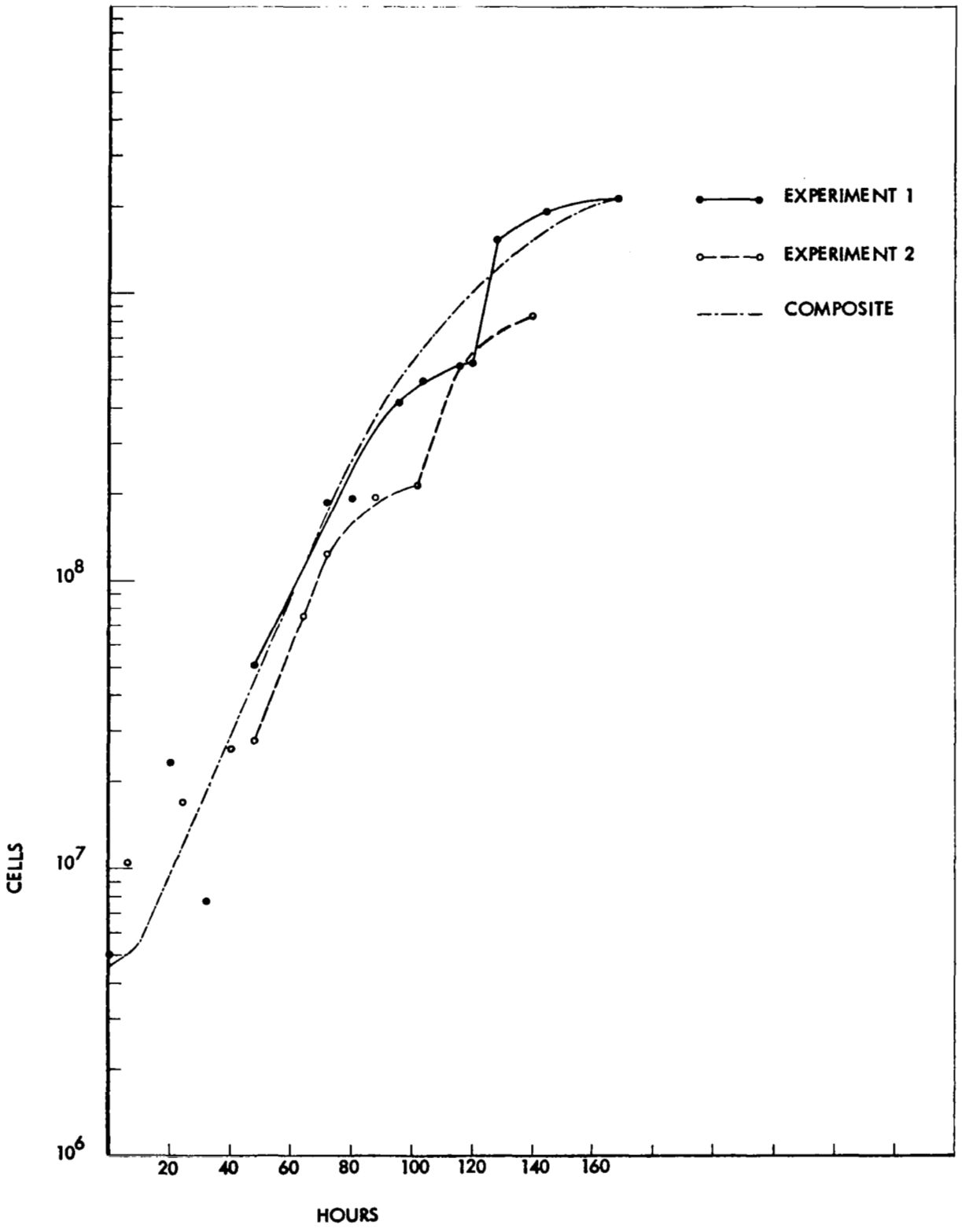

Figure 8. Growth at a_w .95

Table 22

Growth of Halophilic Chromatium at a_w .95, I. First Experiment

	Optical Density		Cells x 10^6	
Hours	$\bar{x}$	σ	$\bar{x}$	σ
0	0	0	4.6	1.0
8	0	0		
24	0.05	0.012	23.2	0.7
32	0.141	0.104	7.7	1.3
48	0.612	0.257	21.9	1.1
52	0.919	0.073		
56	1.49	0.231	50.6	1.5
63	1.55	0.005		
72	2.84	0.701	190	0.2
76	1.98	0.15		
80	2.72	1.21	191	0.7
84	2.44	0.42		
96	1.92	0.47	414	0.6
102	1.79	0.24		
104	2.54	0.52	495	0.2
120	3.35	0.48	574	1.2
128	3.72	0.60	1510	0.5
144	4.27	0.47	1960	0.3
168	10.93	1.35	2290	0.2
192			1490	0.2

Table 23

Growth of Halophilic Chromatium at a_w .95, II, Second Experiment

Hours	Cells x 10^6 $\bar{x}$	σ
0	5.0	0.8
16	10.6	0.2
24	17.0	0.5
40	26.2	0.4
48	27.2	0.7
64	76.6	3.7
72	125	1.1
88	193	-
114	215	0.6
136	575	1.7
140	843	2.1

Section 4

CONCLUSIONS

The ecological situation reported here is unique, not because of essentially one factor limitation, or because of the novelty of the organisms (which have been known for many years[3]), but because of the adaptability of environment to analysis and prediction by theory (phase rule). Specific examples of the applicability of the approach are: 1) definition of the salts in the environment, 2) determination of growth rates and calculation of water activity for saturated systems containing complex solid phases, 3) demonstration of entrapment and survival of organisms within crystals of soluble salts, and 4) prediction of the effect of solid phase transitions on water activity.

Growth has been established as a function of water activity with sodium chloride and sodium carbonate, but not with sodium sulfate, where the relationship is to some other property of the solute. The organism is remarkable in its ability to endure changes in concentration of soluble salts, and in its capacity for growth in dilute and saturated solutions. For this reason, caution must be advocated in assigning this organism to categories based on halophilism. As a photosynthetic bacterium, the pure isolate apparently belongs in Van Niel's[3,12] group of small Chromatium species promoting sulfur formation in the medium.

It seems important to reiterate that if growth occurs in saturated solutions, or if the theoretical limit occurs below or at saturation, the point of stasis would be reached on solid salts in equilibrium with an atmosphere of lower relative humidity than existing over the saturated solutions. This implies that experimentation on growth in the range of water activities between saturation and stasis must be performed using solid salts equilibrated with various relative humidity environments, unless use of salts such as CsCl in solution is possible. In this case, growth must be proven a function of water activity and not of the salt itself.

Growth rates of the halophilic Chromatium at a_w .76 seem consistent with growth localization on a solid phase within the pond. It should be recalled that the pond is analogous to a continuously diluted culture during six months of the year. Although the organisms were isolated from a pigmented layer and Koch's postulates fulfilled, it cannot be stated with certainty that photosynthetic bacteria are the cause of salt deposit pigmentation. Efforts to extract bacteriochlorophyll from the location have been consistently unsuccessful; it was found that the salts present interfered with the method. Improved methodology and determination of carotenoids are indicated.

Conclusions on the correlation of declining growth rates with saturation and increased energy requirement for water uptake seem premature at this point. However, it seems significant that theoretical analysis of the problem has indicated that, in saturated solutions, water does not move osmotically, but must be pumped into the cell. Further work along these lines is necessary, along with an analysis of physiological mechanisms involved.

Hydrate-anhydrate phase transitions should be considered as a possible temperature controlled mechanism for release of water for holophilic growth.

Section 5

ANALYSIS OF RESULTS

This research is perhaps most significant to practical NASA objectives as an example of work with life in an unusual, stressful environment. The results thoroughly emphasize the importance of characterization of the environment as a prerequisite to determination of life by its basic characteristics, organized replication and development, and illustrate the difficulties involved in detecting growth even with well established cultural techniques. For example, log phases in saturated brines were days, not hours, long. Even when the environment is known, and may be simulated, it may require alteration in an organized way to achieve best results.

The data also indicate the care which must be taken in interpreting indications of apparent growth, and in assigning a specific role to an organism in a given environment. For example, growth took place in solutions saturated with salts found in the evaporator ponds and Koch's postulates were fulfilled, but the extent of growth in the natural environment was not determined. Worthy of note here was the interference of environmental constituents with an established analytical method for bacteriochlorophyll.

A second practical aspect of this program is the approach taken to theoretical determination of the ultimate point of limitation of life by water. The central points here are the equilibrium between the energy available for growth and increase and the energy of attraction of water by the environment.

A third practical result is the applicability of phase rule to prediction of the effects of temperature on water activity. Correlation of Martian temperatures and conditions predicted by phase rule for realization of water activities higher than the ultimate might allow determination of areas on Mars most likely to yield samples containing life.

Section 6

PROGRAM PERSONNEL

The following technical personnel participated in this program:

R. W. Tew, PhD (Program Manager)

K. H. Sweeny, PhD

A. T. Yahiro, PhD

P. F. Fan, PhD

D. K. Alford

C. T. Johnson

J. Parkinson

T. Crowe

B. E. Volcani, PhD (Consultant)

Paul Blatz, PhD (Consultant)

Section 7

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APPENDIX A

COMMENTS ON HALOPHILISM

by

Paul Blatz, Ph.D.

California Institute of Technology

The experimental approach to determining the water limitation of life is valuable in that unequivocal results are obtained, but in the present case pertains to only one of a number of possible halophilic experimental subjects. Thus, experimentation should be accompanied by a theoretical approach so that specific results might be analyzed within a more general framework. It is important to determine the correlation between growth limitation of the photosynthetic halophiles by water and the values determined by theory. Before proceeding, however, it is also important to recognize that the halophilic Chromatium is anaerobically photosynthetic, and that this process apparently produces water.

We take the original cell shape to be that of a cylinder 1.1μ long and 1.3μ in diameter. The volume of this cylinder is given by

$$\frac{\pi}{4}\ 1.3^2 \times 1.1\mu^3 = 1.5 \times 10^{-12} \text{cc} \qquad 1)$$

The density of the brine in which the cell either floats or sinks is approximately 1.27g/ml. Since the cell appears to be slightly heavier than the brine, its density may be taken to be 1.30g/ml. Further if we assume that 15 weight % of the cell is organic material (of which protein accounts for 50%, and carbohydrate and lipid for the remaining 50%) and that the cell is in brine of density 1.27g/ml, then it is easy to calculate that the density of the organic material must be 1.50g/ml, in order to yield an overall average cell density of 1.30g/ml. These facts are summarized in Table I. It is useful to state also the composition of the brine. This is presented in Table II.

Thus after 3.23 days a typical cell of volume $1.50\mu^3$ ingests $1.3\mu^3$ of brine of density 1.27g/ml weighing 1.66×10^{-12}g. This brine contains 363.8g of mixed salts per liter of solution. This is presented in Table III.

These salts are dissolved in 902.7g or 57.5 moles of water. In the average cell, which has a volume of 1.30×10^{12} $\mathring{A}^3$, there are thus:

29.9×10^8 molecules NaCl
4.0×10^8 molecules Na_2SO_4
4.9×10^8 molecules Na_2CO_3
403.0×10^8 molecules H_2O

and 1.8×10^8 molecules organic material, with an assumed molecular weight of 150.

Considering first the organic material, we find that one molecule of average molecular weight 150 occupies 1100 $\mathring{A}^3$, and has the dimensions of 11$\mathring{A}$ on a side which is perfectly consistent with the dimensions given for polymer molecules in standard texts on the subject.

Scaling down the brine, we find that a 10$\mathring{A}$ cube count is

3.67 Na^+ ions
2.31 Cl^- ions
.31 $SO_4^=$ ions
.37 $CO_3^=$ ions
31.0 H_2O molecules

On the other hand, a 10$\mathring{A}$ cube of water contains 33-1/3 molecules. Thus, in forming the brine from pure water and mixed crystals, there is an entropy loss attributable to volume contraction, or hydration, and an increase owing to mixing. Of these two the latter effect will predominate. Thus, despite the fact that approximately 30 water molecules can bind approximately four alkali halide molecules without volume change, there is still an entropy increase from molar and charge mixing. In addition there is a strongly exothermic heat of mixing. This implies that, in

order to remove a water molecule from such a concentrated brine, there must be considerable expenditure of activation energy. This activation energy could be calculated if the state of the water molecule during diffusion through the cell membrane were known In view of the fact that the cell also ingests salt, it cannot be assumed that the activated water is free from salt. In order to shed light on this process, it would prove desirable to measure diffusion rates on various electrolytes of various concentration through the cell membrane. This would provide a very valuable set of information.

In the absence of diffusion data, one interesting conclusion can be drawn: The cell ingests 1.3×10^{-12} cc of brine of the composition given in Table II in 2.79×10^{5} sec through an average surface area of $1.6\,\pi \times 1.3 \times 10^{-8}$ cm^2. This corresponds to a flux of

$$q = \frac{1.2 \times 10^{-12}}{2.79 \times 10^{5} \times 1.6\,\pi \times 1.3 \times 10^{-8}} \approx 10^{-10} \quad \frac{cm}{sec} \qquad 2)$$

A typical diffusion constant, for penetration of electrolyte liquids through polymore membranes is of the order of

$$D \approx 10^{-5}\ cm^2/sec \qquad 3)$$

Taking this as the best reasonable figure we have to work with, we find that the concentration gradient needed to promote diffusion to brine into the cell is given by:

$$-\nabla C = \frac{g}{D} = 10^{-5} \left(\frac{\frac{ml}{ml}}{cm}\right) \qquad 4)$$

Now a typical cell has an edge length of 11,000Å. If we assume the thickness of this membrane to be 100Å we find

$$-\nabla C = \frac{\Delta C}{\Delta X} = 10^{-5} \frac{\frac{cc}{cc}}{cm} \qquad 5)$$

and $$\Delta X = 10^{2}Å = 10^{-6}\ cm \qquad 6)$$

so that $$\Delta C = 10^{-11} \frac{cc}{cc} \qquad 7)$$

Thus, only a tiny fluctuation in density of the brine is needed to explain the observed diffusion rate, provided the cell membrane thickness and diffusion constant are each of the right order of magnitude.

In order for the cell to maintain this density gradient, it is necessary to expend energy. There are two possibilities. First, it may expand energy by activating a water and/or salt molecule so that it can travel thru the cell wall (this energy may be considerable-several kilocalories) or it may recover this energy by a metabolic process, which means that only a minute amount of net energy needs to be expended to pump the water/salt into the cell. The other possibility is that the cell does not recover the activation energy and that it is dissipated as heat, since the overall diffusion process requires essentially no energy change if the concentration gradient computed above is correct.

Consider first the case in which the cell does not recover the energy. In this case, there is a danger that the energy provided by the photosynthetic step (and which is now shared by the phytosynthetic anabolic process and the diffusion process) may not be sufficient for both processes. In addition, because of the heat liberated, such a process would have a characteristically high temperature coefficient. Both of these points need investigation.

If, on the other hand, the cell recovers the energy, there is the legitimate question of having to invent a new process by which a diffusing molecule of water can collide with some organic intermediate and give it enough energy to produce some sort of metabolic process. This is presumably a highly speculative suggestion, and without any evidence to the fore must be regarded as improbable.

Thus it is important that both the rate and temperature coefficient of the diffusion through the cell membrane as well as the energetics of the cell metabolism be investigated with a view to deciding the correctness of the second proposed mechanisms.

In addition to providing more information about the thermodynamics of the cell membrane, it will also be necessary to provide more detailed information about the thermodynamics of the brine solution. For example, if the cell membrane is able to activate a water and/or salt molecule and pump it through, the

amount of energy it will have to expend for the activation process will depend on two things: The actual state of the water in the passage through the cell membrane and the actual state of the water in the brine solution. Considering the cell membrane first, if the water remains hydrated to a salt ion which diffuses along with it through the cell membrane, the activation energy will be less than if it diffuses as a free water molecule. On the other hand, the mobility of a water-salt complex will be much less than that of a water molecule which can travel by a Grotthus-type mechanism.

Table I

COMPOSITION OF ASSUMED TYPICAL CELL

	Volume μ^3	Weight, 10^{-12} g	Density, g/cc	Volume Fraction	Weight Fraction
Total Cell	1.50	1.95	1.30	1.00	1.00
Organic Material	.20	.29	1.50	.13	.15
Brine	1.30	1.66	1.27	.87	.85

Table II

BRINE COMPOSITION

	Volume, cc	Weight, g	Density, g/cc	Volume Fraction	Weight Fraction
Brine	1000.0	1266.5	1.2665	1.0000	1.000
Water	902.7	902.7	1.0000	.9027	.713
Mixed Salts	157.0(as crystal) -59.7 = ΔV_{mix}	363.8	2.315(as mixed crystal)	.0973(as hydrate)	.287

Table III

COMPOSITION OF MIXED SALTS IN BRINE*

NaCl	224.0g	or	3.830 moles
Na_2SO_4	73.1g	or	.513 moles
Na_2CO_3	66.7g	or	.619 moles

* Plus minor components.

Similarly, in the brine solution, the free energy of a water molecule will depend on its proximity to a salt ion. If the water diffuses alone, the cell may have enough energy to pick up a water molecule two or three shells of nearest neighbors removed from the salt ion, but not enough to pick one out of the first shell. Thus it will be necessary to compute the free energy as a function of distance from the ion center. This has been done for extremely dilute solution according to the Debye-Huckel presentation, the basic idea behind which is that the ions are distributed around a given cation according to the Boltzmann distribution. From this distribution one calculates the change density in the neighborhood of the selected ion, and then solves Poisson's equation to obtain the electrostatic potential around the ion. On adding the contribution from the translational motion of the ion, one obtains the free energy of the ions in solution, and finally the activity coefficient as a function of concentration. This approach is good only for extremely dilute solution, in which range the water molecules may be assumed to obey Raoult's law. The main conclusion of the Debye-Huckel theory is that the log of the activity coefficient is proportional to the square root of the ionic strength.

For more concentrated solutions, empirical modifications of this relation have been suggested, which make the log of the activity coefficient a function of the square root of the ionic strength. A more rational approach consists in allowing for the formation of ion pairs and even ion quadruplets, etc., as suggested by Bjerrium. Robinson and Stokes extended this idea to its logical conclusion and consider equilibrium between free and bound water being considered as that in the first shell of hydration. They contend that the Debye-Huckel treatment then gives the mean activity coefficient for the hydrated ions, and thus arrive at the expression:

$$\log \gamma_{H_2O\ (25^\circ F)} = \frac{.5092\ z_A\ z_B \sqrt{c}}{1 + .3586\ \overset{\circ}{a} \sqrt{c}} \qquad 8)$$

Where $\overset{\circ}{a}$ is the closest distance of approach between hydrated ions, measured in $\overset{\circ}{A}$.

Now in order to decide whether the cell can activate the free or unbound water, we need an expression for the activity of the water in the cell membrane. This conclusion is based on the following thought. For the brines under discussion the concentration gradient needed to promote diffusion through the wall at the observed rate is negligible. Thus the cell needs to expend no net energy to transport the water. Presumably the same argument holds for the hydrated salt ions. However, the cell does have to activate the water to introduce it into the membrane and then it must recover the energy of activation before it is dissipated as heat. Either that, or it has to have enough energy left over from the photosynthetic process to activate the water without recovering the heat. The way to obtain this is by measuring the diffusion constant and viscosity of the water in the brine, as well as the diffusion rate through the wall. For dilute solution the diffusion constant of the water in the brine is related to the viscosity and the activity by the Nernst equation, given by:

$$\frac{D\eta}{D_o\eta_o} = 1 \frac{e^2 K}{4 Dkt} = 1 \frac{\ln \gamma}{2z-z} \qquad 9)$$

For more concentrated solutions, the writer suggests that the Nernst equation be modified according to the concepts of Stokes and Robinson. Presumably the outcome will look something like the following:

$$\frac{D\eta}{D_o\eta_o} = \frac{1-A\frac{K}{D}}{1+B\frac{K}{D}} \qquad 10)$$

In order to look into this and arrive at a useful solution, more time would be needed than it was possible to expand in this preliminary investigation. The main points of the preceding discussion are summarized below.

In conclusion, the observed diffusion or (really) ingestion rate is consistent with an extremely minute concentration gradient across the cell membrane. This gradient is so minute that the cell must actually pump the brine through the wall; the calculated gradient of 10^{-11} being much less than that observed in many osmotic processes. Both the rates and temperature coefficients of this pumping reaction for brines of various compositions should be measured.

Prior to pumping, the cell must activate a water molecule, which we now assume (following Stokes and Robinson) is either bound in the first hydration shell, or free in the second and higher shells. It is further assumed that the cell does not recover the activation energy which is dissipated as heat. Thus it is important to provide information regarding how much free energy is available for the activation process. This is the difference between the available from the photosynthetic process, and that used up by the first step of the phytosynthetic process.

The activation energy can be taken as proportional to the difference between the logs of the activity coefficient of the water in brine, for which information is available, and the activity coefficient of the water in the cell membrane, which information is not available. But this can be obtained by measuring the diffusion coefficient of the water through the cell as suggested above (Item 2), and by calculating the viscosity, and then by applying a modified Nernst equation to calculate the activity. In lieu of calculating the viscosity in the cell wall, it is suggested that it be taken to be the same as that in the brine which can be measured.

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