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The role of glycerol in the nutrition of halophilic archaeal communities: a study of respiratory electron transport

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Abstract

Respiratory electron transport activity in the Dead Sea and saltern crystallizer ponds, hypersaline environments inhabited by dense communities of halophilic archaea and unicellular green algae of the genus *Dunaliella*, was assayed by measuring reduction of 2-(*p*-iodophenyl)-3(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to INT-formazan. Typical rates obtained were in the order of 5.5–17.7 nmol INT reduced h^{-1} per 10^6 cells at 35° C. In Dead Sea water samples, respiratory activity was stimulated more than two-fold by addition of glycerol, but not by any of the other carbon compounds tested, including sugars, organic acids, and amino acids, or by addition of inorganic nutrients. Stimulation by glycerol had a half-saturation constant of 0.75 μM. A similar respiratory activity was also found when Dead Sea water samples were diluted with distilled water and incubated in the light. As *Dunaliella* cells did not reduce INT, it is suggested that photosynthetically produced glycerol leaking from the algae is the preferred carbon and energy source for the development of halophilic archaea in hypersaline environments. In samples from saltern crystallizer pond stimulation of INT reduction by glycerol was much less pronounced, probably because the community was less severely carbon-limited.

Keywords: Respiratory electron transport; Tetrazolium salts; Halophilic archaea; Dead Sea; Salterns; Glycerol

1. Introduction

Halophilic archaea (genera *Halobacterium, Haloferax, Haloarcula*, and *Halococcus*) are abundant in hypersaline lakes in which salt concentrations exceed 250 g l^{-1}, such as the Dead Sea, the Great Salt Lake, Utah, and crystallizer ponds of solar salterns all over the world. Community densities of 10^7–10^8 halophilic archaea per ml brine are not exceptional in such environments [1,2].

Halophilic archaea are heterotrophs, that grow in culture at the expense of simple organic compounds such as amino acids and certain carbohydrates. Little is known on their mode of nutrition in nature [1,2], but it has often been postulated that glycerol may be a key nutrient. Glycerol is a suitable carbon and energy source for most or all Halobacteriaceae [2,3], and it can be expected to be available as it serves as an osmotic stabilizer in species of *Dunaliella*, the unicellular green alga that is often found in high numbers in the same hypersaline environments in

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which halophilic archaea thrive. *Dunaliella* accumulates glycerol intracellularly in molar concentrations, and blooms of *Dunaliella* are often accompanied by mass development of halophilic archaea [1,2,4,5].

It was stated more than 20 years ago that the halophilic archaea "are proteolytic or saccharolytic and probably derive most of their carbon from glycerol and cell protein produced by *Dunaliella*" [6]. However, only recently measurements of glycerol turnover and metabolism in hypersaline brines yielded the first evidence for the validity of such a statement. Two experimental approaches led to a recognition of the possible importance of glycerol as a key metabolite, connecting autotrophic and heterotrophic metabolism in hypersaline environments: estimations of in situ glycerol concentrations and its turnover time [3], and studies on the fate of low concentrations of glycerol added to Dead Sea or saltern brines [7,8]. In situ glycerol concentrations were found to be below 0.8–1.4 μM, while maximal glycerol uptake rates were high (160–426 and 193–303 nmol l^{-1} h^{-1} for Dead Sea and saltern brines, respectively), and estimated turnover times for glycerol were short (0.45–3.3 h and 2.6–7.2 h, respectively) [3]. Additional evidence for the importance of glycerol in the carbon cycle of hypersaline environments came from the finding that glycerol is transformed in part into organic acids (acetate, D-lactate, and pyruvate) by cultures of halophilic archaea, and the same acids were detected when the biota of the Dead Sea and saltern crystallizer ponds were incubated with micromolar concentrations of radioactively labelled glycerol [8]. Formation of ^{14}C-labelled pyruvate, lactate, and acetate was shown to occur both in Dead Sea brine incubated in the light with $^{14}CO_2$, and in cocultures of *Dunaliella parva* and *Haloferax volcanii* [7].

In the present study, we assessed the respiratory electron transport activity of halophilic archaeal communities in the Dead Sea and in solar saltern ponds by measuring reduction of 2-(*p*-iodophenyl)-3(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to INT-formazan, and measured the effect of addition of different carbon sources and other manipulations. As documented here, these measurements led to additional insights on the role of glycerol as a key compound in the carbon cycle in hypersaline ecosystems.

2. Materials and methods

2.1. Sampling sites

Dead Sea surface water was collected at the shore near Ein Gedi, and samples from different depths were pumped through a hose at the deepest point of the lake, about 8 km east of Ein Gedi. Samples of brines of different salinity were collected from evaporation ponds of the solar salterns at Eilat, Israel. Sampling sites were described earlier [9].

2.2. Estimation of algal and bacterial numbers

Bacteria were enumerated microscopically, using a Petroff-Hausser counting chamber and a microscope equipped with phase contrast optics. When necessary, the bacteria were concentrated by centrifugation (15 min at 12000 × *g*) prior to counting. *Dunaliella* cells were counted by filtering samples through Millipore filters (5 μm pore size), and counting cells on the filter under a 16 × objective [10]. Numbers were calculated from the average cell number per field and the field diameter.

2.3. Tetrazolium reduction assays

In the standard assay for 2-(*p*-iodophenyl)-3(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) reduction, 10 ml portions of Dead Sea or saltern brine in 20 ml sterile test tubes were supplemented with 0.2 ml of a solution of 2 g l^{-1} INT (Sigma), followed by incubation in the dark at 35° C for periods between 2.5 and 6 h. In most cases experiments were started within 2 h of sampling. The procedure was modified with respect to incubation time, temperature, and addition of sterile distilled water or different organic and inorganic nutrients, as indicated. Subsequently, the contents of the tubes were filtered through glass fiber filters (Whatman GF/C, 25 mm diameter). Filters were transferred to 1.5 ml plastic centrifuge tubes, and extracted for 1 h with 1 ml ethanol. After removal of the filters and centrifugation, the absorbance of the extracts was measured at 488 nm. Control experiments included zero-time controls, and incubation of brine from which the bacteria had been removed by filtration through GF/C glass fiber filters. Calculations of the

INT reduction rate were based on a specific absorbance of 1.42×10^4 $M^{-1} cm^{-1}$ for INT-formazan [11].

2.4. Bacterial and algal strains and culture conditions

Halobacterium sodomense ATCC 33755 was grown in 100 ml portions in 250 ml Erlenmeyer flasks in a rotatory shaker at 35° C in medium containing (g l^{-1}): NaCl, 125; $MgCl_2.6H_2O$, 160; K_2SO_4, 5; $CaCl_2.2H_2O$, 0.1; yeast extract, 1, casamino acids, 1, and soluble starch, 2. Formation of bacteriorhodopsin was induced by growing cultures under oxygen limitation in the light [12]. Cultures of *Haloferax volcanii* ATCC 29605 and *Haloarcula marismortui* ATCC 43049 were grown as described earlier [8].

Axenic cultures of *Dunaliella parva* isolated from the Dead Sea were grown in the light (white fluorescent tubes, incident irradiance 40 μmol quanta $m^{-2} s^{-1}$) at 35° C in medium containing (g l^{-1}): NaCl, 125; $MgCl_2 \cdot 6H_2O$, 25; KCl, 1.34; $MgSO_4 \cdot 7H_2O$, 0.075; Na_2CO_3, 0.02; $NaNO_3$, 1.5; KH_2PO_4, 0.039; Fe-citrate, 0.006; citric acid, 0.006, Na_2EDTA, 0.001, and trace elements (final concentrations in mg l^{-1}: H_3BO_3, 0.08; $MnCl_2 \cdot 4H_2O$, 2.9; $ZnSO_4 \cdot 7H_2O$, 1.2; $Na_2MoO_4 \cdot 2H_2O$, 0.22, and $CuSO_4 \cdot 5H_2O$, 0.02).

2.5. Glycerol assays

Glycerol was assayed enzymatically by measuring reduction of NAD^+ to NADH in the presence of glycerol dehydrogenase (EC 1.1.1.6). Reaction mixtures (final volume 1 ml) contained 25 μmol Na-py-

Table 1
Effect of addition of different organic and inorganic compounds on the rate of INT reduction by the archaeal community in Dead Sea water and Eilat saltern brines

Analysis	Sample				
	Dead Sea			Eilat salterns	
				pond 301	pond 200
	17 August 1994	12 September 1994	4 October 1994	22 September 1994	
Salinity (σ_{25}) [a]	228	231	233	240	100
Bacteria ml^{-1}	1.6×10^7	1.4×10^7	1.0×10^7	2.1×10^7	ND
Dunaliella ml^{-1}	580	290	55	650	0
INT reduction rate [b] (nmol $l^{-1} h^{-1}$) with:					
No addition	121 ± 7	76 ± 4	55 ± 1	372 ± 8	168 ± 10
Glycerol	288 ± 9	161 ± 1	102 ± 2	566 ± 11	187 ± 8
D-Glucose	149 ± 5	86 ± 1	41 ± 2	418	186 ± 3
Trehalose	137 ± 3	ND	ND	ND	ND
Mannitol	120 ± 1	N.D	ND	ND	ND
Na-acetate	118 ± 6	ND	37 ± 2	390 ± 12	184 ± 5
Na-succinate	ND	90 ± 3	46 ± 2	380 ± 10	192 ± 2
Na-pyruvate	170 ± 3	ND	52 ± 2	404 ± 9	178 ± 8
Na-L-lactate	148 ± 6	ND	ND	ND	182 ± 5
Li-D-lactate	146 ± 4	94 ± 2	ND	503 ± 14	172 ± 10
Na-L-malate	ND	81 ± 2	ND	ND	ND
Na-glycolate	ND	75 ± 1	41 ± 1	380 ± 6	172 ± 5
Na-L-glutamate	165 ± 2	ND	ND	ND	ND
Casamino acids	170 ± 8	84 ± 2	60 ± 2	535 ± 17	178 ± 9
Yeast extract	197 ± 4	120 ± 10	69 ± 1	436 ± 10	188 ± 10

[a] Salinity was expressed as σ_{25}, the density departure from that of distilled water at 25° C, as measured using a hydrometer.
[b] INT reduction was measured in Dead Sea surface water (sampled 17 August and 12 September 1994 at the shore near Ein Gedi, and on 4 October 1994 in the center of the lake) and in Eilat saltern brines of different salinities. Measurements were performed in the dark at 35° C. Values (mean ± standard deviation) are based on duplicate or triplicate determinations. Organic compounds were added from concentrated solutions to a final concentration of 10 mg l^{-1}.
ND, not determined.

rophosphate buffer, pH 10, 5 units of *Cellulomonas* sp. glycerol dehydrogenase (Sigma), and 1.5 mg β-NAD. After 4 h incubation at 30° C the absorbance at 340 nm was measured against blanks in which the enzyme was omitted from the reaction mixture.

3. Results

3.1. INT reduction measurements in Dead Sea and saltern brines

Dead Sea water samples reduced INT to INT-formazan at rates between 55–121 nmol l^{-1} h^{-1} without added substrate (Table 1), or 5.5–7.6 nmol h^{-1} per 10^6 microscopically recognizable bacteria at 35° C. The activity can be attributed to respiration by the archaeal community. Axenic cultures of the alga *Dunaliella parva*, which was present in the samples examined (between 55 and 580 cells ml^{-1}) did not reduce INT, neither in the light, nor in the dark, while cultures of halophilic archaea examined (*Hb. sodomense, Hf. volcanii, Ha. marismortui*) readily reduced INT. No significant INT reduction was observed in brine samples filtered through GF/C filters. GF/C filters were previously shown to effectively retain the bacteria present in hypersaline brines [3]. The amount of formazan formed increased linearly with time, and the INT concentration used (40 mg l^{-1}) was found saturating. Addition of higher concentrations often led to the formation of precipitates. Addition of glycerol greatly stimulated INT reduction, and a concentration of 10 mg l^{-1} caused an increase of between 96 and 110 percent in the rate of formazan formation (Table 1). Microscopic examination of Dead Sea surface water samples incubated for 6 h in the presence of INT and 0.1 mM glycerol (see below) showed the presence of formazan granules in 75–80% of the particles recognizable as bacteria in Dead Sea surface water (12 September 1994).

The depth distribution of INT reduction rate in the water column of the Dead Sea showed a good correlation with the bacterial counts (Fig. 1). In the mixed layer above the thermocline/pycnocline located at a depth of 16–20 m, rates varied only little (between 40–59 nmol INT reduced h^{-1} l^{-1} or between 3.7–5.7 nmol h^{-1} per 10^6 cells at 35° C). Below a depth of

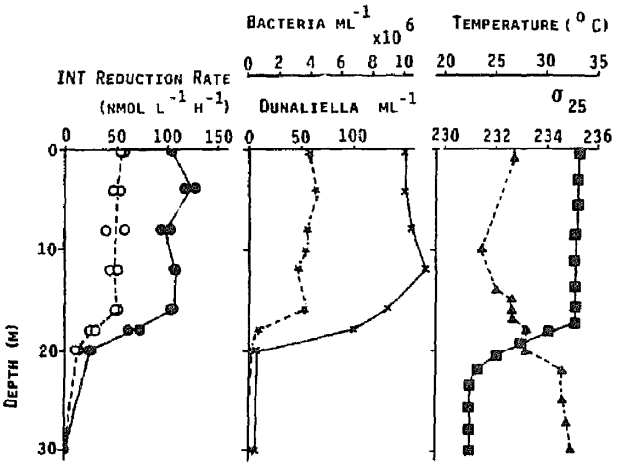

Fig. 1. Vertical distribution of INT reduction rate, measured at 35° C in the absence (○) and presence (●) of 0.1 mM added glycerol (left panel), as correlated with the community density of bacteria (×), and *Dunaliella* cells (★) (middle panel), and with temperature (■) and salinity, expressed as σ_{25}, the density departure from that of distilled water at 25° C (▲) in the water column of the Dead Sea on 4 October 1994 (right panel). Data on salinity and temperature were kindly supplied by D.A. Anati.

20 m bacterial numbers were very low, and INT reduction was hardly detectable. Standard INT reduction assays were performed at 35° C, a temperature close to that of Dead Sea surface water in summer. Rates were found to be strongly influenced by temperature, maximal INT reduction rates being reached at 60° C (Fig. 2).

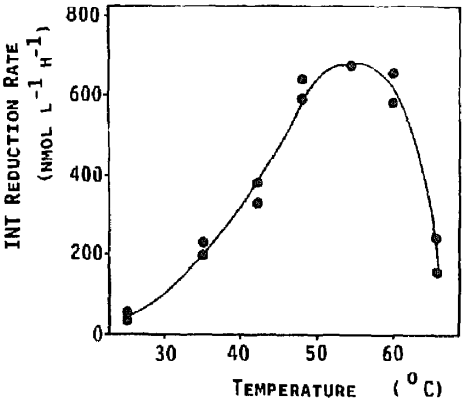

Fig. 2. Temperature dependence of INT reduction by the microbial community of Dead Sea water. Dead Sea surface water, sampled 17 August 1994, was incubated in the dark at different temperatures in the presence of 40 mg l^{-1} INT and 0.1 mM glycerol, and the formation of INT-formazan was measured after 2.5 h.

INT reduction rates in the same order of magnitude as found in the Dead Sea were measured in the NaCl-saturated crystallizer ponds at Eilat (372 nmol l^{-1} h^{-1}, or 17.7 nmol h^{-1} per 10^6 cells) (Table 1). Intracellular formazan granules were observed in 85–90% of the microscopically recognizable bacteria in this brine after 6 h incubation in the presence of INT and glycerol (22 September 1994).

3.2. Effect of addition of organic substrates and other compounds on INT reduction rates

To obtain information on the factors limiting archaeal respiratory activity, as measured by INT reduction, and on the nature of the substrates and conditions that may stimulate this activity, the effect of the addition of a variety of organic and inorganic compounds on INT reduction rates in Dead Sea and saltern brines was investigated. Whereas glycerol was found to greatly stimulate INT reduction (Table 1, Fig. 1), hardly any stimulation, if at all, was found by any of the many other organic compounds tested, including sugars, amino acids, and other potential substrates. A slight stimulation was found when yeast extract was added, probably due to the fact that yeast extract contains small concentrations of glycerol. Our enzymatic analysis of Difco yeast extract for glycerol yielded a glycerol content of 0.25% by weight. The specific stimulating effect of glycerol on INT reduction in Dead Sea and saltern brines was found already at very low concentrations, and the

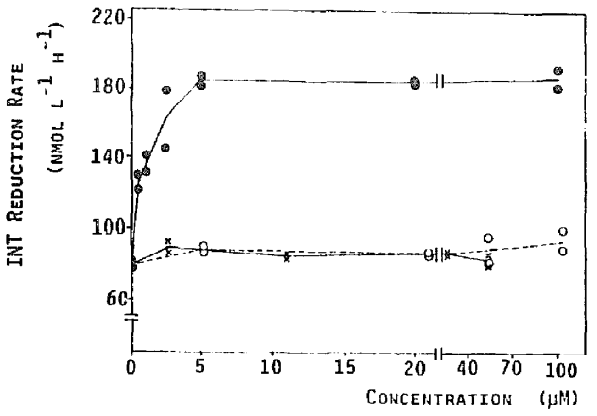

Fig. 3. Effect of the concentration of added glycerol (●), D-glucose (×), and Li-D-lactate (○) on the rate of INT reduction by the archaeal community in Dead Sea water. Dead Sea surface water, sampled 17 August 1994, was incubated in the dark at 35° C with 40 mg l^{-1} INT and organic substrates as indicated, and the formation of INT-formazan was measured after 5 h.

estimated half saturation constant of about 0.75 μM shows the high affinity of the archaeal community for glycerol. Other potential substrates, such as glucose and D-lactate, did not affect the rates at any of the concentrations tested (Fig. 3). Stimulation by glycerol was even more pronounced when the salinity of the brine was reduced by dilution with distilled water.

No increase in INT reduction rates was measured upon addition of inorganic nutrients such as ammonium or phosphate salts, added at a concentration of 1 μM. Addition of the electron transport phosphory-

Table 2
Effect of light and dilution with distilled water on the rate of INT reduction by the archaeal community in Dead Sea water

Dilution (%)	INT reduction rate (nmol l^{-1} h^{-1}) [a]					
	17 August 1994		12 September 1994		4 October 1994	
	Light	Dark	Light	Dark	Light	Dark
0	124 ± 6	99 ± 3	65 ± 2	46 ± 3	34 ± 1	35 ± 1
5	ND	ND	96 ± 2	70 ± 3	40 ± 3	50 ± 2
10	162 ± 20	91 ± 2	103 ± 3	75 ± 4	50 ± 3	54 ± 2
15	ND	ND	ND	ND	54 ± 5	48 ± 5
20	ND	ND	121 ± 5	87 ± 3	ND	ND
30	ND	ND	121 ± 4	93 ± 5	ND	ND

[a] INT reduction was measured in 10 ml portions of Dead Sea surface water, to which different amounts of sterile distilled water had slowly been added. Details on the properties of the brine samples are given in Table 1. Measurements were performed at 35° C in the dark or in the light (white fluorescent tubes, incident irradiance 100 μmol quanta m^{-2} s^{-1}). Values (mean ± standard deviation) are based on three or four replicate determinations.
ND, not determined.

lation uncouplers FCCP (carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone) (5 μM) or 2,4-dinitrophenol (1 mM) did not result in increased INT reduction rates; FCCP even caused a slight inhibiton.

An increase in INT reduction rate, comparable with that elicited by addition of glycerol, could be obtained by slowly diluting Dead Sea water samples with distilled water, followed by incubation in the light (Table 2). As *Dunaliella* cells did not reduce INT, neither in the light, nor in the dark, and as the only substrate found to stimulate INT reduction by the archaeal community was glycerol, the most likely interpretation of the results obtained is that part of the glycerol produced in the light by the *Dunaliella* population leaks out of the cells, and becomes available to the archaea present. The effect of dilution with distilled water is then probably due to the fact that the salinity of the sample was too high to enable high rates of photosynthesis by the *Dunaliella* cells. It has been documented earlier that *Dunaliella* can grow in Dead Sea water only after reduction of its salinity by dilution [4,5,10]. The slight stimulation found in several cases in the dark INT reduction rates after dilution may be due to a direct effect of the addition of distilled water on the *Dunaliella* cells, either causing leakage of glycerol in an attempt to adjust the osmotic pressure and/or cell volume [13,14], or lysis of some of the cells, in spite of the care taken during the slow dilution. It is thus suggested that excess glycerol produced photosynthetically by the algae is the main carbon and energy source for the development of halophilic archaea in the Dead Sea. Dead Sea water samples collected on 4 October 1994 contained very few *Dunaliella* cells, and accordingly the INT reduction rates in the light were not greatly different from those in the dark (Table 2).

To verify that light did not directly stimulate respiratory activity in halophilic archaea, INT reduction by suspensions of Dead Sea isolates of halophilic archaea (*Hf. volcanii*, *Ha. marismortui*, *Hb. sodomense*) in Dead Sea water was measured in the light and in the dark. No stimulation by light was found in any case, not even in a bacteriorhodopsin-rich culture of *Hb. sodomense*.

In samples from a saltern crystallizer pond, inhabited by a dense community of halophilic archaea and large *Dunaliella salina* cells, specific stimulation of INT reduction by glycerol did occur, but the increase in rate of formazan formation (46%) was less pronounced than in the Dead Sea samples, probably because the community was less severely carbon-limited. Accordingly, INT reduction rates in the light and in the dark did not differ significantly (data not shown). In a saltern evaporation ponds with a lower salinity, at which halophilic and halotolerant eubacteria rather than archaea are active, hardly any stimulation of INT reduction by glycerol or any of the other carbon compounds was observed (Table 1).

4. Discussion

Reduction of tetrazolium salts has been used in different ways to assess planktonic microbial activity. One often used method involves collection and homogenization of the cells, followed by measurement of INT reduction in the presence of potential electron donors such as NADH, NADPH, and succinate, in an attempt to obtain potential rates of electron transfer [11,15–18]. In another approach, used in the present study, INT is added to the untreated water sample, with the purpose of estimating endogenic rates of electron flow through the bacterial respiratory chain. The nature of the electron donors is unknown in this case, and the possibility cannot be excluded that the addition of the electron acceptor may significantly alter the in situ rate of electron transport. We tested the effect of addition of compounds that may limit bacterial activity in the sample on INT reduction rates, and obtained information on the physiological state of the bacterial community in hypersaline environments.

INT reduction has also been used to estimate the percentage of the bacterial community active in respiration in marine samples, by incubating water samples with the tetrazolium salt, followed by microscopic examination of the cells for the presence of formazan granules [19]. A similar assay performed here with Dead Sea and Eilat saltern crystallizer brines showed that the method can easily be adapted to hypersaline brines, and the percentage of respiring cells in the samples was estimated to be in the order of magnitude of 75–90%.

Of all organic compounds tested as potential substrates, only glycerol was found to greatly stimulate

INT reduction by the biota in the Dead Sea and in saltern crystallizer brines. In these environments, essentially all heterotrophic activity can be attributed to halophilic archaea, and eubacteria do not contribute significantly [1,2,9]. Among the substances that caused no or little increase in INT reduction were pyruvate, D-lactate and acetate, compounds which are formed by partial oxidation of glycerol by halophilic archaea, both in culture, and in their natural habitats [7,8]. Also glycolate, which may be formed as a result of algal photorespiration, had no influence, as expected because glycolate has never been reported to be used by halophilic archaea. Even a compound such as succinate, which may donate electrons directly to the electron transport system, did not have much effect. The present study thus confirms the hypothesis that excess glycerol produced photosynthetically by *Dunaliella* cells is the preferred carbon and energy source for the development of halophilic archaea in hypersaline environments. This hypothesis is supported by earlier studies of estimations of in situ glycerol concentrations and its turnover time [3], and studies in the fate of radioactively labelled glycerol or CO_2 added to Dead Sea or saltern brines was followed [7,8]. The halophilic archaeal communities are thus well adapted to the efficient use of low concentrations of glycerol.

Addition of glycerol to hypersaline brines dominated by halophilic archaea gave rise to an increase in INT reduction rate of up to 110% in the case of Dead Sea brines, and 46% in saltern crystallizer ponds. The affinity of the cells for glycerol proved very high, half of the maximum stimulation being achieved at a concentration as low as 0.75 μM, a value in the same order of magnitude of the actual glycerol concentrations estimated to exist in these hypersaline environments [3]. This value is also close to the typical K_m values reported for glycerol kinase (1–2 μM) [20], which is the enzyme by which glycerol is expected to enter cell metabolism in halophilic archaea. Glycerol kinase activity was shown to be present constitutively in all halophilic archaea examined [21]. Oxidation of glycerol by glycerol dehydrogenase probably does not occur to a significant extent, as the activity was detected only in *Halobacterium salinarium* and in *Hb. cutirubrum*, types of halophilic archaea that do not occur in high numbers, if at all, in the hypersaline brines examined [2], and no activity could be demonstrated in *Hb. saccharovorum*, *Hb. sodomense*, and in representatives of the genera *Haloferax* and *Haloarcula* [21]. Moreover, glycerol dehydrogenase characteristically has a high K_m value for glycerol, in the order of 2–10 mM [20].

Dunaliella cells produce glycerol to serve as an intracellular osmotic solute, and this is possible thanks to the very low permeability of its cell membrane to glycerol [22,23]. Most other biological membranes are highly permeable to glycerol, and no explanation has yet been brought forward for the special behavior of the *Dunaliella* cell membrane in this respect. However, small amounts of glycerol may leak out of healthy *Dunaliella* cells. We found glycerol concentrations on the order of 0.1–0.7 mM in the supernatant of exponentially growing cultures of *D. parva* [7], and the stimulation of INT reduction observed when Dead Sea samples were incubated in the light suggests that leakage of photosynthetically produced glycerol may occur in natural communities of *Dunaliella*, supplying the archaeal community with its preferred carbon and energy source. Other culture studies of different *Dunaliella* strains have shown that the often large amount of dissolved organic carbon excreted (up to 1–5% of the photosynthetically fixed carbon, and up to 11% when ammonia served as nitrogen source) contains only very small amounts of glycerol, if at all [24]. Whatever the nature of the organic compounds released by *Dunaliella* communities in nature, development of blooms of halophilic archaea in hypersaline lakes always occurs when *Dunaliella* cells are present in sufficiently high numbers.

When *Dunaliella* cells are subjected by hypotonic stress as a result of dilution with less saline water, large amounts of glycerol may leave the cell, as documented in culture studies [13,14]. In this study a small extent of stimulation of INT reduction in the dark was often observed upon diluting Dead Sea water with small amounts of distilled water (Table 2). The possibility cannot be excluded that a small number of cells also did burst as a result of the treatment.

The finding that addition of glycerol greatly stimulated INT reduction by the biota of the Dead Sea proves that, at least at the time of the investigation, the activity of the archaeal community was limited

by the amount of available carbon source. The much smaller extent of stimulation of INT reduction by added glycerol in samples from the saltern crystallizer pond (pond 301), suggests that carbon limitiation was probably much less severe in the saltern crystallizer ponds, due to the presence of a dense and active community of large *Dunaliella salina* cells. Indications for relatively fast growth rates of halophilic archaea in these ponds, with estimated doubling times between 1.2 and 12.2 days, have been derived in the past from measurements of thymidine incorporation by these brines [9].

No evidence was found to support the conclusion from an earlier study, that lack of phosphate limited the halophilic archaea in the Dead Sea [25]. Lowering the salinity of the sample somewhat stimulated INT reduction, and the effect of a subsequent addition of glycerol was much more pronounced than in undiluted samples. Thus, the fact that the archaea live in the Dead Sea in the presence of about 1.8 M magnesium and 0.4 M calcium ions, much above the concentrations optimal for their growth [26,27], may also be a major factor determining the heterotrophic activity. Availability of phosphate is, however, a condition required for the development of *Dunaliella* in the Dead Sea [4,10], and phosphate thus influences indirectly the community size and activity of the halophilic archaea.

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