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Preservation of ATP in Hypersaline Environments

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High concentrations of particulate ATP were found in the anoxic brines of the Orca Basin and East Flower Garden, Gulf of Mexico. Other measurements indicative of growth and respiration suggested that the microbial community in the brines was inactive, but somehow the ATP associated with the cells persisted. Conceivably, when cells growing just above the interface sank into the brine, the increased osmotic stress could elicit an osmoregulatory response resulting in increased ATP. It was also possible that hydrolytic enzymes were inactivated, resulting in the preservation of ATP. Experiments in which a culture of marine bacteria was suspended in menstrua of different salinities comparable to those found across the Orca Basin interface revealed that as salinity increased, ATP increased three- to sixfold. Within 24 h the ATP fell to its initial level and remained at that concentration for 3 days, at which time the experiment was terminated. In contrast, the control suspensions, at a salinity of 28‰ (grams per liter) had 1/10th of the initial ATP concentration when the experiment was ended. Cells were also exposed to killing UV irradiation, enabling us to demonstrate with absolute certainty that cellular ATP could be preserved. At the end of the experiment, the viable component of the population was reduced by orders of magnitude by UV irradiation, but the ATP levels of the cells suspended in brine did not decrease. In certain environments it appears that the conventional analytical tools of the microbial ecologist must be interpreted with caution.

Measurements of ATP have been used as a valuable means to estimate the biomass of viable microbes in the oceans (11–14, 17, 19, 21–23). The value of such estimates depends on the fact that ATP is immediately hydrolyzed upon the death of a microbial cell and does not persist in association with cell remains or detritus (14). However, environments that inhibit enzyme activity, especially that of ATPases, could conceivably contain large amounts of particulate ATP (P-ATP) associated with nonviable cells that have accumulated over long periods of time. In these environments, the use of P-ATP as an indicator of living microbial biomass would be inaccurate and misleading.

High levels of P-ATP have been reported in the anoxic brine of the Orca Basin in the Gulf of Mexico (26). However, rates of [^{3}H]uridine incorporation into RNA and [^{14}C]acetate utilization suggested that little or no growth was occurring immediately below the oxic-anoxic interface. A similar situation was found in another brine at the East Flower Garden, also in the Gulf of Mexico, where the undecomposed remains of zooplankton (9) and the apparent lack of microbial growth (P. A. LaRock, R. Lauer, T. Bright, and E. Powell, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, N61, p. 254) suggested an inactive microbial community despite P-ATP concentrations of 188 ng/liter. We considered that the discrepancy between ATP and activity measurements could be explained if the brine environment preserved ATP by preventing its hydrolysis.

The occurrence of dissolved ATP has been documented, but we can eliminate this possibility because normally membrane filtration is used to concentrate the microbial material, and as such the dissolved component would not be significant.

Preliminary experiments with potato apyrase revealed that simply by adding increasing amounts of NaCl, the rate of ATP hydrolysis could be retarded and eventually stopped at a concentration of 360‰ (grams per liter). We also found that when a culture of marine bacteria was suspended in artificial brine, the cells remained viable for some time, although they did not grow. The results of our subsequent experiments are presented in this paper. We conclude that P-ATP derived from bacteria that grow at the oxic-anoxic interface is preserved when the bacteria sink into the brine.

MATERIALS AND METHODS

The culture used was *Serratia rubidaea* (ATCC 19279), a marine bacterium extensively used in laboratory experiments. The culture was carried on 2216 marine agar (Difco Laboratories, Detroit, Mich.). For all salt-effect experiments, 2216 broth was prepared.

The experiment was a repeated-measures factorial design (two levels of irradiation by three levels of salt). Over a time course, we measured cell viability, incorporation of [^{3}H]adenine into nucleic acids, and ATP concentrations. UV radiation was used as a treatment because bacteria might only be inhibited by increased levels of salt, but UV light would ensure the death of very nearly all the cells. Thus, any P-ATP remaining after irradiation would indicate its preservation in dead cells.

Six treatments were tested: concentrations of brine salts at 28, 150, and 240‰ with exposure to UV radiation and the same concentrations with no exposure to UV light. The salt concentrations reflect values observed across a salinity gradient in the Orca Basin (30). *S. rubidaea* was grown on 2216 plates and harvested with sterile artificial seawater to yield an inoculum containing 8×10^8 cells per ml. Twenty-four 250-ml flasks, each containing 50 ml of 2216 broth, were randomly assigned to six treatments; thus, there were four replicates of each treatment. A 1-ml sample of the inoculum was added to each of the 24 flasks, yielding an initial bacterial density of 1.6×10^7 cells per ml. The flasks were maintained on a rotary shaker at 125 rpm. Next, brine salts were added incrementally over 2.5 h to a concentration of 150 or 240‰. The brine salts were added in increments since

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preliminary experiments had shown that addition of the total amount of salt at once resulted in osmotic shock with erratic fluctuations in ATP levels. The artificial brine salts were formulated based on analysis of the East Flower Gardens brine by Brooks et al. (5): 985 g of NaCl per liter, 2.6 g of K_2SO_4 per liter, 4.3 g of $CaSO_4$ per liter, 4.3 g of $MgSO_4$ per liter, 3.8 g of Na_2SO_4 per liter. No comparable analysis is available for the brine in the Orca Basin. For treatments involving UV light, each flask was emptied into a separate sterile petri dish on a rotary shaker table. The cell suspension (4 mm deep) was exposed to a General Electric G15T8 germicidal lamp at a 5-cm distance for 1 min while being agitated. This procedure reduced the viable cell count by a factor of 10^5 (28‰) to 10^6 (150 and 240‰). The exposed cell suspensions were then poured into new sterile flasks and incubated together with the unirradiated samples on a rotary shaker table.

Samples to determine the uptake of [^{3}H]adenine, ATP content, and cell viability were taken at the start of the experiment and 2.5 (after salt addition), 3 (after UV exposure), 5, 24, 48, and 72 h after inoculation.

Incorporation of radioactive adenine into nucleic acids was measured by a modification of the method of Kennell (24). For each sample, a 1-ml portion was transferred to a 10-cm^3 syringe to which a 25-mm membrane filter (0.45-μm pore size) had been attached. Each sample was then incubated with 2 μCi of [^{3}H]adenine (NET-063; New England Nuclear Corp., Boston, Mass.) for 30 min. After isotope incubation, 5 ml of cold 10% trichloroacetic acid was added to each syringe, mixed with the sample, and allowed to stand for 15 min. The samples were then filtered. The syringes were opened and refilled with 10 ml of cold trichloroacetic acid. Five minutes later, the nonprecipitated contents of the syringe were expelled through the filter. This was followed by three rinses with 95% ethanol to remove residual trichloroacetic acid, after which the incorporated activity was determined by liquid scintillation counting with quench correction.

For ATP determinations, 0.5 ml of each sample was injected into boiling Tris buffer, extracted for 5 min, and then frozen for later assay by the luciferin-luciferase reaction (15). Before assay, the 150 and 240‰ extracts were diluted 6- or 10-fold, respectively, to reduce residual salt interference (21) to a level equivalent to that in 28‰ extracts. After appropriate dilutions, 0.5 ml of extract was combined with 0.1 ml of purified luciferase enzyme (Dupont Instruments, Inc.) and the emitted light was read with an Aminco Chem-Glow photometer. Results were converted to ATP concentrations based on standard curves.

In our protocol, we chose to extract ATP from cells in suspension, rather than from filtered cells, to avoid stress resulting from filtration (20). The resultant ATP concentrations, therefore, were the sum of dissolved and particulate components (2). At the end of the experiment, we assessed the contribution of dissolved ATP by filtering samples from each treatment and assaying the supernatant for ATP. In all instances the results were at the background level.

Cell viability was determined by plate counts, using only one of the quadruplicates of each treatment. For each sampling period, the particular flask used was chosen randomly. A 0.1-ml sample was withdrawn and serially diluted in sterile seawater, and 0.1 ml of the appropriate dilutions was plated in quadruplicate on 2216 marine agar.

The effects of salinity and UV light on adenine incorporation and ATP concentration were assessed by completely randomized, two-factor analyses of variance (ANOVAs). Four ANOVAs were performed in all to assess adenine incorporation at 5 and 72 h and ATP concentration at 5 and 72 h. The statistical null hypotheses were that exposure to UV, the salinity of the medium, and the interaction of these factors have no effect on adenine incorporation or ATP concentration. Residuals and normal scores were examined, and the data were $\log_{10}$transformed to meet the assumptions of the ANOVA. After the ANOVA, Tukey's honestly significant difference (HSD) test was calculated to compare treatment means by pairs.

RESULTS

The effects of experimental treatments on cellular ATP, ability to incorporate [^{3}H]adenine, and viability of the culture are shown in Fig. 1 and 2. At 28‰, adenine incorporation by cells not exposed to UV light increased during the first 5 h (Fig. 1A, open circles), corresponding to a nearly 10-fold increase in cell number (Fig. 2, open triangles). After 5 h, cell number showed virtually no change (Fig. 2), accompanied by a decrease in adenine incorporation. Because the percentage of the cellular dry mass constituted by RNA decreases substantially with declining growth rate (16), reduced adenine incorporation from 5 to 48 h presumably reflects unbalanced growth associated with the transition to the stationary phase. Adenine incorporation by irradiated cells was constant through 5 h (Fig. 1A, solid circles), reflecting the effects of exposure to UV light. Incorporation of radioisotope increased 10-fold by 24 h, corresponding to recovery and growth of cells that survived irradiation (Fig. 2, solid triangles). At 48 and 72 h, the irradiated and unirradiated treatments exhibited approximately the same cell density. The ATP concentration of the unirradiated samples declined approximately 10-fold over the course of the experiment (Fig. 1B, open circles). Irradiation, however, resulted in an immediate fivefold increase in ATP values even though viable cell numbers declined by 10^5 (Fig. 1B, solid circles). The ATP concentration gradually declined to the levels found in the unirradiated sample at 72 h.

At a salinity of 150‰, the unirradiated samples maintained a relatively uniform rate of adenine incorporation throughout the experiment (Fig. 1C, open circles). Correspondingly, plate counts of viable bacteria (Fig. 2, open squares) also remained fairly constant. Cells exposed to UV light exhibited a 100-fold decrease in adenine incorporation after exposure, followed by a further but more gradual decline in uptake from 5 to 24 h (Fig. 1, solid circles). In parallel fashion, viable cells decreased from 10^9 to 10^2/ml after exposure and remained essentially constant through 72 h (Fig. 2, solid squares). Changes observed in ATP concentrations were essentially the same for both unirradiated and UV-exposed cells (Fig. 1D). Initial concentrations were about 3 ng/ml, but after brine salts were added, values increased to 25 ng/ml in both treatments. With time, ATP concentrations decreased in both treatments. In the unirradiated samples, the decline was less abrupt and the terminal ATP concentration was much greater than in the 28‰ samples.

At a salinity of 240‰, irradiated and unirradiated treatments exhibited virtually no difference in the incorporation of adenine (Fig. 1E). During the addition of brine salts, there was a large decrease in incorporation followed by a gradual decline over the remainder of the experiment. ATP concentrations were identical for both samples (Fig. 1F) and initially increased from 3 to 17 ng/ml. Levels of ATP then decreased and remained constant at 3 ng/ml from 24 to 72 h.

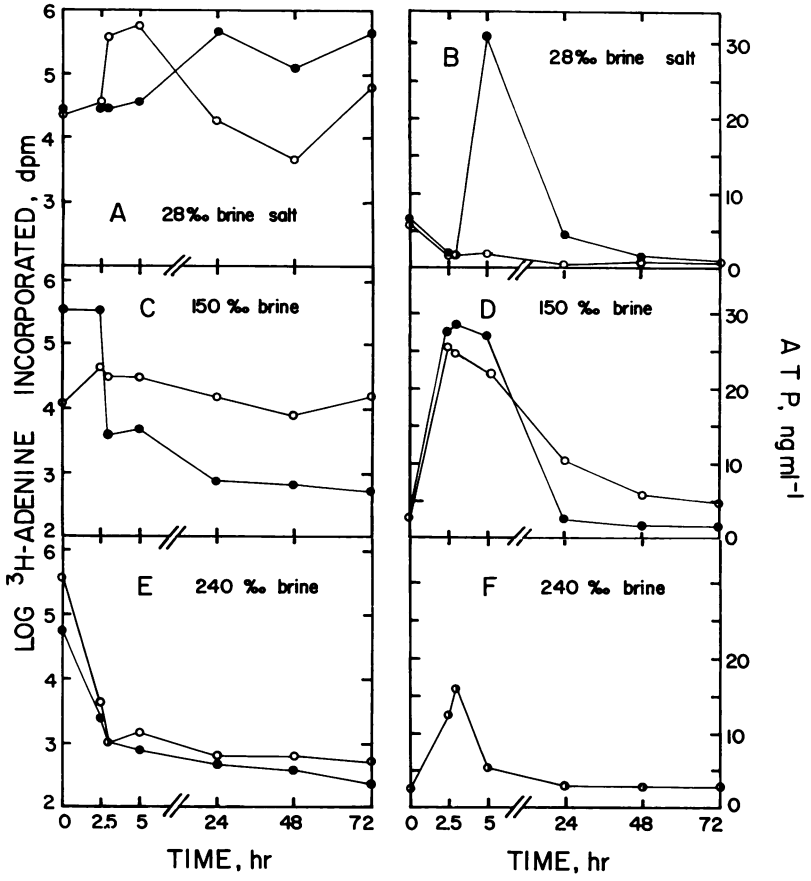

FIG. 1. [³H]adenine incorporation (A, C, and E) and ATP concentrations (B, D, and F) in suspensions of *S. rubidaea* at three salinities. Symbols: ●, exposure to UV radiation; ○, no exposure.

For irradiated samples, there was essentially no net loss of ATP despite the absence of viable cells (Fig. 2, solid circles). Cells not exposed to UV light demonstrated an overall decline in viability from 10^9 to about 10^5 cells per ml at the end of the experiment (Fig. 2, open circles).

ANOVA performed separately on the 5- and 72-h data indicated that incorporation of [³H]adenine was significantly affected by salt concentration, exposure to UV irradiation, and the interaction of these factors ($P < 0.001$ for all three). Uptake ranged over 4 orders of magnitude, and with one exception, the rank order of means was the same at 5 h as it was at 72 h (Table 1). As the salinity of the medium increased, incorporation of adenine decreased. At any one salinity, exposure to UV light further reduced adenine incorporation. The exception occurred at 72 h in a medium of 28‰, when the bacterial suspensions exposed to UV radiation exhibited greater incorporation than their unexposed counterparts. This difference reflects the growth of the survivors after UV exposure as seen in the enumeration data (Fig. 2).

Similarly, the concentrations of ATP observed at 5 and 72 h were affected by salinity, UV exposure, and the interaction of these treatments ($P < 0.001$ for all three). At 5 h there was no significant difference between the irradiated and unirradiated samples at either the 150 or 240‰ salinities (Table 2). At 28‰, however, there was a highly significant increase in ATP in the irradiated sample. At 72 h, after the perturbation in ATP had passed, there was no significant difference between the irradiated and unirradiated samples at either the 28 or 240‰ salinities. Mean ATP levels in the unirradiated samples at 150‰, however, were more than twice those in the irradiated samples. ATP levels were higher in the brine samples than in the 28‰ samples. ATP was also found in the presence of no viable cells (UV, 240‰).

DISCUSSION

The significant findings of our work are: (i) exposure of bacteria to elevated salt levels or UV radiation results in an abrupt increase in ATP that may raise cellular concentrations fivefold; (ii) the ATP levels in a normal growth medium will decline with time to 1/10 their initial values as the culture ages; (iii) bacteria immersed in brine salts do not show a loss of ATP in the long term and retain their original ATP concentrations throughout the experiment, even though viability may be decreased by exposure to salts or UV radiation. In a related, less complex experiment, we found that P-ATP concentrations of *S. rubidaea* remained unchanged for 18 days after stabilization subsequent to the addition of brine salts and irradiation by UV light.

Changes in ATP levels have been used in a variety of circumstances to determine bacterial response to an antagonistic agent or environmental change. The ATP content of bacterial cells has been found to decrease significantly as cultures increase in age (6, 11, 17), are starved (18, 31, 35), are cooled (7), or are exposed to arsenate (32). A reduction in ATP levels has been used to test the efficacy of disinfection processes (33) or as an indicator of toxicity (25).

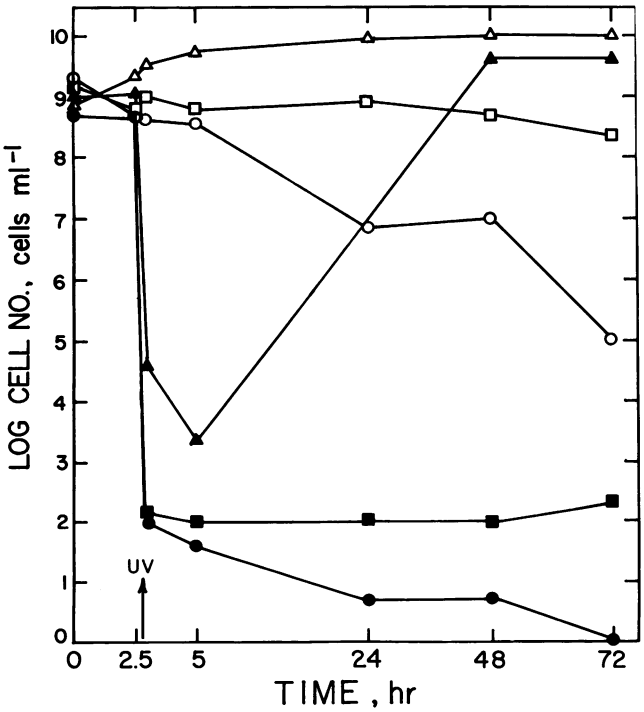

FIG. 2. Viable cell number of *S. rubidaea* suspended in menstrua of different salinities, with and without UV irradiation. Symbols: △ and ▲, 28‰ salinity; □ and ■, 150‰ salinity; ○ and ●, 240‰ salinity. Solid symbols indicate exposure to UV radiation, and open symbols represent no exposure. Data were not obtained at 24 h for irradiated cells at 28‰.

Considerable research with adenine nucleotides has been done to elucidate the feedback mechanism of energy charge (see reference 1), but there is scant information concerning the energy demands imposed on a cell in response to changes in its physiology. In contrast to the work cited above, the ATP content of bacteria exposed to increased osmotic pressure or UV-induced DNA damage increases nearly fivefold. Presumably this increase reflects (i) an attempt to offset increased osmotic pressure and (ii) processes that the cell is undertaking to cope with, or remedy, the effects induced by the antagonists, namely repair of UV-induced pyrimidine dimers.

The principal factors that determine osmotolerance are (i) the resistance of the enzymes within a cell to the solutes present and (ii) the ability of the cell to produce compatible solutes that will permit continued enzyme activity and

TABLE 1. Tukey's HSD test on incorporation of [^{3}H]adenine by *S. rubidaea*

Treatment	Mean (dpm) at:	
	5 h after inoculation	72 h after inoculation
UV, 240‰	741[a]	278[a]
No UV, 240‰	1,517[a]	484[a]
UV, 150‰	5,396[a]	503[a]
No UV, 150‰	28,564[b]	17,325[b]
UV, 28‰	37,805[b]	463,418[c]
No UV, 28‰	654,919[c]	93,040[d]

[a–d] Within a column, means with the same superscript letter are not significantly different in Tukey's HSD test at a family error rate of 0.05.

TABLE 2. Tukey's HSD test on concentration of ATP in *S. rubidaea*

Treatment	Mean (ng/ml) at:	
	5 h after inoculation	72 h after inoculation
No UV, 28‰	2.02[a]	0.42[a]
UV, 28‰	33.92[b]	0.42[a]
No UV, 150‰	22.04[c]	4.15[b]
UV, 150‰	26.89[b,c]	1.47[c]
No UV, 240‰	5.46[d]	2.51[d]
UV, 240‰	5.10[d]	2.55[d]

[a–d] Within a column, means with the same superscript letter are not significantly different in Tukey's HSD test at a family error rate of 0.05.

balance the external osmotic pressure to avoid dehydration (10). In *Escherichia coli*, for example, it is proposed that an osmosensory protein embedded in the envelope triggers the synthesis of compounds capable of affecting the osmotolerance of the cell (27). Metabolically controlled compatible compounds contributing to osmotolerance include free amino acids such as glycine, alanine, proline, and β-alanine and their derivatives, glycerol and other polyols (36), and dimethylsulfide (34). Since the synthesis of these compounds requires energy, it is not unreasonable to hypothesize that the rapid increase in ATP we observed as the salt mixture was added to the cell suspension reflects attempts to produce some compatible solute in response to the elevated osmotic stress.

The damage inflicted by UV radiation is predominantly photoinactivation of DNA by the formation of thymine dimers (29). Repair and recovery can be accomplished by photoreactivation or photoreversal. In the absence of light, dimer excision and reconstructive repair of the damaged DNA strand can be mediated enzymatically. In *E. coli*, DNA damage or inhibition of DNA replication results in expression of a group of cellular events called the SOS system (4). The SOS response includes inhibition of cell division, stimulation of error-prone repair, and reactivation of prophages. A series of regulated genes must be activated to perform their respective repair functions and then repressed as DNA repair is terminated. A model detailing the regulation of the SOS system has been proposed (28).

Barbe et al. (4) irradiated *E. coli* with UV light, observed a twofold increase in levels of cellular ATP 20 to 30 min after exposure, and proposed a relationship between changes in concentration of ATP and expression of the SOS system. Working with mutants of *E. coli*, they demonstrated a balance between production of ATP via degradation and hydrolysis of ATP related to cleavage of a gene repressor (3). Immediately after exposure to UV light, ATP is produced faster than it can be used. With time, ATP consumption becomes more rapid and ATP returns to its preirradiation level.

In our experiment, concentrations of ATP in the 28 and 150‰ irradiated samples (Fig. 1B and D) increased sixfold. We cannot specify the exact nature of this response in *S. rubidaea*, but ATP certainly would be required to synthesize enzymes necessary for replacement of cellular components damaged by irradiation (8).

Our results indicate that high levels of ATP, accompanied by impaired physiological capability, should be interpreted cautiously, as there are conditions under which ATP per se can be misleading. Furthermore, we found that the test culture responded to the salt and UV stress by producing elevated concentrations of ATP, a result that has not been

previously reported. In view of the findings we report here, we conclude that elevated ATP values associated with anoxic brines or, possibly, other extreme environments may in fact be associated with dead or severely stressed cells.

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