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Hydrocarbon Biodegradation in Hypersaline Environments

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When mineral oil, hexadecane, and glutamate were added to natural samples of varying salinity (3.3 to 28.4%) from salt evaporation ponds and Great Salt Lake, Utah, rates of metabolism of these compounds decreased as salinity increased. Rate limitations did not appear to relate to low oxygen levels or to the availability of organic nutrients. Some oxidation of L-[*U*-^{14}C]glutamic acid occurred even at extreme salinities, whereas oxidation of [1-^{14}C]hexadecane was too low to be detected. Gas chromatographic examination of hexane-soluble components of tar samples from natural seeps at Rozel Point in Great Salt Lake demonstrated no evidence of biological oxidation of isoprenoid alkanes subject to degradation in normal environments. Some hexane-soluble components of the same tar were altered by incubation in a low-salinity enrichment culture inoculated with garden soil. Attempts to enrich for microorganisms in saline waters able to use mineral oil as a sole source of carbon and energy were successful below, but not above, about 20% salinity. This study strongly suggests a general reduction of metabolic rate at extreme salinities and raises doubt about the biodegradation of hydrocarbons in hypersaline environments.

Microbiological studies of hypersaline environments are generally lacking, and indeed recent reviews demonstrate the meagerness of our understanding of the microbiology of extremely saline systems (14, 17, 18). Systematic studies of Great Salt Lake (17) and of the Dead Sea (12) demonstrated that bacteria are the major components of an extremely large biomass in saline lakes (10^6 to 10^8 bacteria per ml by direct count; Secchi depth, 0.5 to 4 m; viable counts in Great Salt Lake yielded 10 to 20 times fewer cells than observed by direct microscopy). Although extreme salinity has obviously restricted microbial diversity, recent biological and geochemical evidence also suggests the presence of microbial processes common to conventional lakes (i.e., sulfate reduction, methane genesis) in Great Salt Lake (17) and Dead Sea (14) sediments. Extensive decomposition of naturally occurring (wood, tar balls, and organic debris in Great Salt Lake [17]) or artificially added (filter paper, wood, fish, and insects in the Dead Sea [12]) organic matter by heterotrophic microflora indigenous to these lakes was not observed. However, results of experiments on hypersaline lagoons (7) and evaporation ponds (4) suggested that heterotrophic activity was greater at lower salinity. Geochemical evidence of microbial weathering of hydrocarbons in a Canadian oil field also correlated with decreasing salinity of associated formation waters (2). The effect of extreme salinity on the rates of microbial processes in hypersaline environments has not been well studied.

Our interest in the metabolism of microorganisms indigenous to Great Salt Lake arose from studies on environmental limitations to hydrocarbon metabolism in lakes (19). Since petroleum has been mined from, and tar seeps are known to occur in, Great Salt Lake (6, 8), we extended our studies to examine the effects of salinity on hydrocarbon metabolism.

MATERIALS AND METHODS

Sample collection and field measurements. Water samples were collected on 14-15 July 1975 in glass or Nalgene sample bottles by direct immersion. Except in enrichment cultures, glassware was not sterilized since we assumed that the extreme salinity of samples would prevent the activity of contaminants from nonsaline sources. Approximate locations of samples are given in Fig. 1. Samples 146-1, 146-4, and 148-1 were collected by boat; sample 148-1 was collected within the sheltered Little Valley Harbor. Sample 145-4 was collected at the shore at Silver Sands Beach peninsula. Samples 143-1, 143-4, 144-1, 144-4, and 145-1 were collected at the shores of various evaporation ponds of the Morton Salt Company Saltair Plant, where water from the south end of Great Salt Lake is concentrated to remove salts. Sample 150-1 was collected at Yogurt Spring, a slightly thermal spring (44°C) of seawater salinity located between Utah Highway 83 and a dismantled railroad bed at the southern base of Little Mountain, downstream from the source at a location where the water had cooled to 33°C.

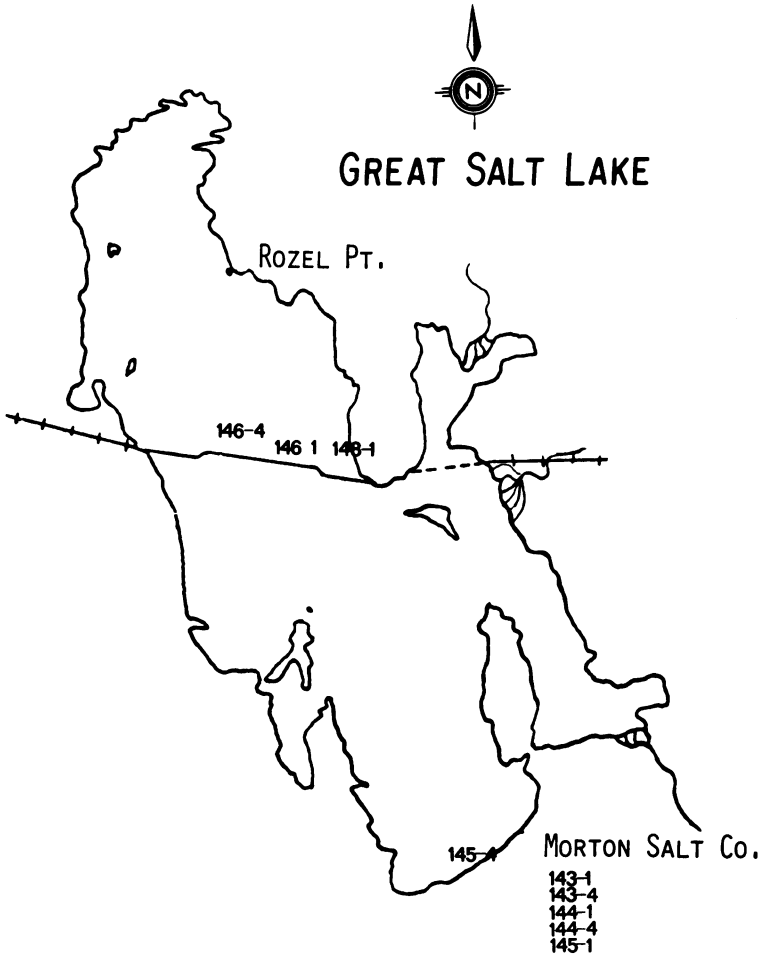

FIG. 1. *Location of samples collected in and near Great Salt Lake.*

To collect samples at desired salinity, approximate salinity was measured in the field by using a hydrometer. Water temperature was measured with a mercury thermometer. Dissolved oxygen was measured chemically in the field by the acid modification of the Winkler assay (1).

Tar samples were collected at Rozel Point (Fig. 1). A sample of beached tar was obtained in a Whirlpak plastic bag (NASCO) on 19 June 1975 at the shore where water was in contact with tar just east of a land-fill pier extending into the lake; the sample was transferred in September 1975 to a solvent-cleaned, foil-capped glass vial. A fresh tar sample was collected for us in November 1975 in a solvent-cleaned foil-capped glass vial from the first well to the east of the land-fill pier mentioned above. Both samples were stored frozen until analysis in June 1976.

Chemical measurements. Salinity was determined in the laboratory by weighing 10-ml water samples in tared volumetric flasks and is expressed as percentage of salt (wt/vol). pH measurements were made by using a Corning model 12 pH meter and a glass combination electrode.

Hydrocarbon and glutamate metabolism in natural samples. Rate studies were initiated on 16 July 1975. The methods for measuring oxygen uptake during the metabolism of mineral oil (Fisher Scientific Co.; to approximately 0.6 g/liter) and monosodium glutamate (45 mg/liter) were the same as reported by Ward and Brock (19), except for the addition of 100 μg of P per liter as KH_2PO_4 and 300 μg of N per liter as NH_4Cl from sterile pH 7 stock solutions (100× concentrated) to prevent nutrient limitation during incubation. Bottles containing samples were incubated in the dark at ambient temperature. Dissolved oxygen was monitored regularly by using a model 51-A oxygen meter and a model 5420 oxygen electrode (Yellow Springs Instrument Company). The meter and probe were calibrated above distilled water at ambient temperature and pressure before making readings. Differences in sensitivity of the probe due to salinity were corrected later by division of the measured oxygen value by a sensitivity factor derived empirically by relating the dissolved oxygen concentration of samples of known salinity (NaCl) measured by the oxygen probe and chemically by the acid modification of the

Winkler method (as above). Oxygen demand of control bottles not amended with substrates was subtracted, and the results are the average of duplicates. Average variation between duplicates was about 0.2 mg of O_2 per liter. If O_2 became depleted, samples were reaerated by bubbling air through bottles for 10 min.

Oxidation of [1-^{14}C]hexadecane (Amersham/Searle) to $^{14}CO_2$ was done as in Ward and Brock (19), using 40-ml samples supplemented to provide nitrogen and phosphorus as above. Oxidation of L-[U-^{14}C]glutamic acid (Amersham/Searle; 290 mCi/mmol, specific activity) to $^{14}CO_2$ was done as above using 0.1 μCi per 40-ml sample. When samples were reaerated (as above), a CO_2 trap was used to ensure complete recovery of the isotope. Errors in counting efficiency were corrected, and data are presented as average disintegrations per minute after subtraction of radioactivity in formaldehyde-killed controls.

Enrichment cultures from natural samples. Two strategies for enrichment of mineral oil-oxidizing microorganisms were employed using medium containing: $(NH_4)_2SO_4$, 1.3 g; KH_2PO_4, 0.28 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $CaCl_2 \cdot 2H_2O$, 0.07 g; and 1 ml of trace element solution (5) per liter of distilled water. One drop of mineral oil (Fisher, white, heavy) was added per 10 ml of enrichment medium. In one type of enrichment, 1 ml of sample was diluted in 9 ml of sterile salts medium (pH 7) in which salinity had been adjusted to 8.5, 15, 20, or 26% NaCl. To provide for growth factors and to maintain indigenous salt balances of water samples, a second enrichment was made at each station by adding 9 ml of sample to 1 ml of 10× concentration sterile salts medium. All enrichments were initiated in the field and incubated at ambient temperature. Growth was determined visually at the oil-water interface and by microscopy.

Chemical analysis of tars. Approximately 200-mg subsamples of tar were loaded onto a glass column (about 20-mm diameter) packed with 40 cm of silica gel (40 to 140 mesh) overlaid with 8 cm of Al_2O_3. Hexane-, benzene-, and methanol-soluble materials were successively eluted with about 250 ml of solvent. Solvents were removed by rotoevaporation, then by passing under a stream of N_2, and finally allowed to evaporate completely in a foil-capped vial overnight at ambient temperature. Vials containing these samples were weighed; the vial weight was subtracted to determine the weight of hexane, benzene, and methanol solubles, and the difference was taken as the weight of asphaltene material. The hexane-soluble components were redissolved in hexane and injected into a Hewlett-Packard model 5830A gas chromatograph with a stainless steel SCOT column (50 ft; ca. 15.24 m) of OV-101 (Perkin-Elmer) and with a flame ionization detector. Temperature was programmed to increase by 4°C/min from 110 to 280°C and to hold for 60 min at 280°C. Injection temperature was 210°C. The carrier gas was helium at a flow rate of 4 ml/min. The identification of major peaks was by coinjection of standards and by gas chromatography and mass spectrometry using a Varian model 1400 gas chromatograph (same column as above) coupled to a DuPont model 491 mass spectrometer interfaced with a PDP 11 computer.

Biodegradation of tar by a soil enrichment culture. The same mineral salts solution used for enrichment cultures described above (except for the substitution, at 1 ml/liter of medium, of a solution containing 7.0 g of $MnCl_2 \cdot 4H_2O$, 1.0 g of $ZnCl_2$, 1.0 g of $CuCl_2 \cdot 2H_2O$, and 10.0 g of $FeCl_3 \cdot 6H_2O$ per liter of distilled water for the trace elements solution) was supplemented, with approximately 1 g per 100 ml of medium, with tar collected from the well pipe at Rozel Point. The mineral salts-tar medium was inoculated with 1 ml of a 10^{-2} dilution of garden soil in the same medium and incubated on a rotary shaker at 30°C for 4 months. After removal of the aqueous medium and three rinses of the residual tar with distilled water, the tar was extracted in chloroform and transferred to a tared vial, and the chloroform was removed by evaporation. Fractionation of the tar was as described above. Hexane-soluble components were analyzed by gas chromatography by using the same gas chromatograph with a 30-m glass SE30 SCOT column with helium as the carrier gas at a flow rate of 4 ml/min. Temperature was programmed to increase at 2°C/min from 100 to 275°C. Injection temperature was 270°C. Identification of major peaks was by coinjection of standards and comparison with chromatograms of fresh tar.

RESULTS

Chemical and physical parameters. Table 1 presents the data on salinity, temperature, pH, and dissolved oxygen content for all water samples. Samples varied over a range of 3.3 to 28.4% salts by weight. Temperature and pH ranges were relatively small (24 to 33°C and pH 7.8 to 8.35), and these parameters were not considered variables. Dissolved oxygen decreased with increasing salinity and was considered a possible variable.

Hydrocarbon metabolism at varying salinity. Oxygen uptake during the metabolism of mineral oil added to water samples of varying salinity is shown in Fig. 2. Because of high oxygen demand, presumably due to decomposition of brine shrimp, sample 145-4 was reaerated as shown by arrows in Fig. 2. The rate of mineral oil metabolism was lower in higher-salinity samples. At 20.4% and higher salinities, oxygen uptake during mineral oil oxidation could not be detected. Reaeration of high-salinity samples (as indicated by arrows along the abscissa in Fig. 2) did not result in stimulation of mineral oil oxidation, indicating that reduced oxygen levels apparently did not explain the lack of oxidation. Addition, to 1 mg/liter, of a complex organic compound mixture (1 g of yeast extract, 0.5 g of proteose peptone, and 0.5 g of Casamino Acids per 100 ml of water) did not stimulate mineral oil oxidation, suggesting that lack of organic nutrients did not explain the lack of mineral oil oxidation in highly saline samples.

The metabolism of hexadecane was also studied by measuring $^{14}CO_2$ released after the addition of [1-^{14}C]hexadecane to samples of varying

TABLE 1. *Physical and chemical parameters of samples and results of enrichment for mineral oil-oxidizing microorganisms*

Salinity (%)	Sample no.	Temp (°C)	pH	mg of O_2 per liter	Type of enrichment[a]	Growth
3.3	150-1	33	8.2	11.7	A	+
11.2	145-4	29	8.2	4.2	A	+
					B + 8.5%	+
12.2	145-1	29	8.35	3.9	A	+
					B + 8.5%	+
13.6	144-4	27	8.2	2-2.5	A	+
					B + 15%	+
					B + 8.5%	+
17.2	144-1	25.5	8.2	2.4	A	+
					B + 20%	+
20.4	143-4	27	8.0	2.0	A	0
					B + 15%	+
					B + 20%	0
25.8	143-1	24	7.8	1.5	A	0
					B + 20%	0
26.8	146-4	24	7.85	1.4	A	0
					B + 26%	0
27.4	148-1	27	7.9	1.8	A	0
					B + 20%	0
28.4	146-1	25	7.8	2.0	A	0
					B + 26%	0

[a] A, 9-ml sample + 1 ml of 10× concentrated basal medium; B, 1-ml sample + 9 ml of basal medium containing the indicated percentage of NaCl.

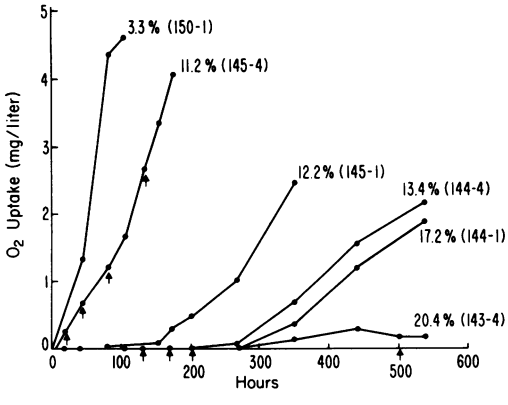

FIG. 2. *Oxygen uptake during mineral oil oxidation in water samples of varying salinity. Oxygen uptake of unsupplemented controls has been deducted. Arrows along the curve labeled 145-4 indicate reaeration of sample 145-4, and arrows along the abscissa indicate points of reaeration of samples of greater than 20.4% salinity. For clarity, data for samples 143-1, 146-1, 146-4, and 148-1 are omitted, since they closely resembled the data of sample 143-4.*

salinity. The rate of hexadecane oxidation was lower in higher-salinity samples (Fig. 3). Reaeration (arrows in Fig. 3, sample 148-1) did not stimulate hexadecane metabolism.

Metabolism of glutamate at varying salinities. As a control for positive heterotrophic growth, glutamate metabolism was also examined in samples of varying salinity. As demonstrated in Fig. 4, the rate of oxygen uptake during glutamate metabolism was also severely retarded in samples of higher salinity. Using a more sensitive radiometric method, we were able to demonstrate that, although the rate of glutamate metabolism was slower, significant oxidation to CO_2 occurred in even the most saline samples (Table 2).

Enrichment of mineral oil-oxidizing microorganism. The results of enrichments from samples of varying salinity are presented in Table 1. In the negative enrichments, there was no growth after incubation times as long as 5 months. In samples of salinity 17.2% or less, all enrichments were successful. Although enrichment in a sample of 20.4% salt using a medium

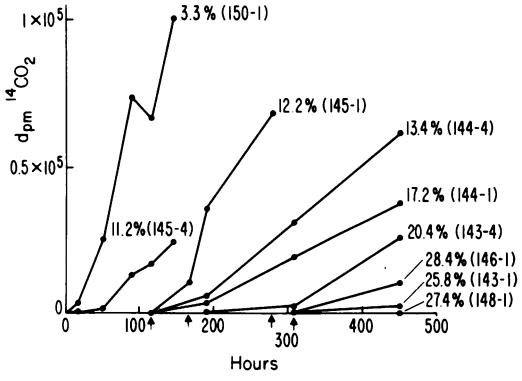

FIG. 3. *$^{14}CO_2$ produced during the metabolism of [1-^{14}C]hexadecane in water samples of varying salinity. Data are averages of duplicates after subtraction of disintegrations per minute of $^{14}CO_2$ in formaldehyde controls. Arrows indicate points of reaeration (sample 148-1).*

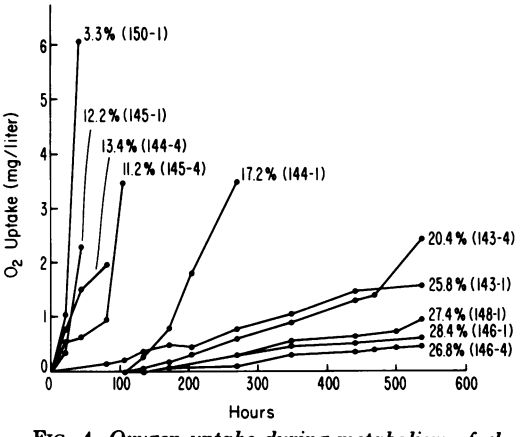

FIG. 4. *Oxygen uptake during metabolism of glutamate added to water samples of varying salinity. Oxygen uptake of unsupplemented controls has been deducted. Samples 146-1, 146-4, and 148-1 were reaerated at the times indicated by arrows in Fig. 2.*

adjusted to 15% NaCl was successful, enrichments of the same sample at near-natural salinity or in basal medium with 20% NaCl were unsuccessful. One enrichment in basal medium plus 20% NaCl (sample 144-1) was positive. No enrichments of higher salinity were positive.

Chemical analysis of tar samples. The percentage by weight of hexane, benzene, methanol, and residual asphaltene fractions of tars collected at the well pipe and on the beach at Rozel Point are given in Table 3. Hexane and benzene fractions comprised a small proportion of tars, and it was difficult to note major differences between these fractions in well pipe and beached tar. The more viscous beached tar contained less methanol-soluble material and more asphaltene compounds than did the well pipe tar, and this may indicate loss of volatile materials from the methanol fraction possibly related to exposure to physical and chemical weathering conditions.

Gas chromatographic analysis of the hexane fractions of the two tars is shown in Fig. 5. Although the source of the two tars is not known to be the same, the striking similarity of the hexane-soluble fractions suggests a common source. The predominant aliphatic hydrocarbons are pristane, phytane, and several steranes as determined by coinjection of standards and mass spectral evidence. The similarity of the hydrocarbon components of the two tars also suggests that no biological alterations occurred during the period of exposure to hypersaline conditions. A soil enrichment culture demonstrated that some portions of the hexane-soluble components of the well pipe tar were amenable to degradation under nonsaline conditions. In particular, the amount of phytane was lowered, as suggested by a pristane/phytane ratio of 2.58 for the biodegraded tar, as compared to pristane/phytane ratios of 0.47 and 0.39 for the well pipe and beached tars, respectively.

DISCUSSION

Our results clearly demonstrate that the rate of hydrocarbon and glutamate metabolism by

TABLE 2. *Percentage of added L-[U-^{14}C]glutamic acid (0.1 μCi or 200,000 dpm) recovered as $^{14}CO_2$ by 40-ml samples of varying salinity*[a]

Salinity (%)	Sample	% $^{14}CO_2$
3.3	150-1	68[b]
11.2	145-4	83[c]
12.2	145-1	90[d]
13.6	144-4	70[e]
17.2	144-1	44[e]
20.4	143-4	48[c]
25.8	143-1	46[e]
26.8	146-4	56[e]
27.4	148-1	40[e]
28.4	146-1	54[e]

[a] Data obtained from averages of duplicates after subtraction of disintegrations per minute of $^{14}CO_2$ in formaldehyde controls.
[b] Incubation, 143 h.
[c] Incubation, 165 h.
[d] Incubation, 279 h.
[e] Incubation, 450 h.

TABLE 3. *Composition of Great Salt Lake tar samples*

Source	% in solvent fraction			
	Hexane	Benzene	Methanol	Residual[a]
Well pipe	1.1	4.3	64.3	30.3
Beached	2.8	7.8	32.9	56.5

[a] By difference.

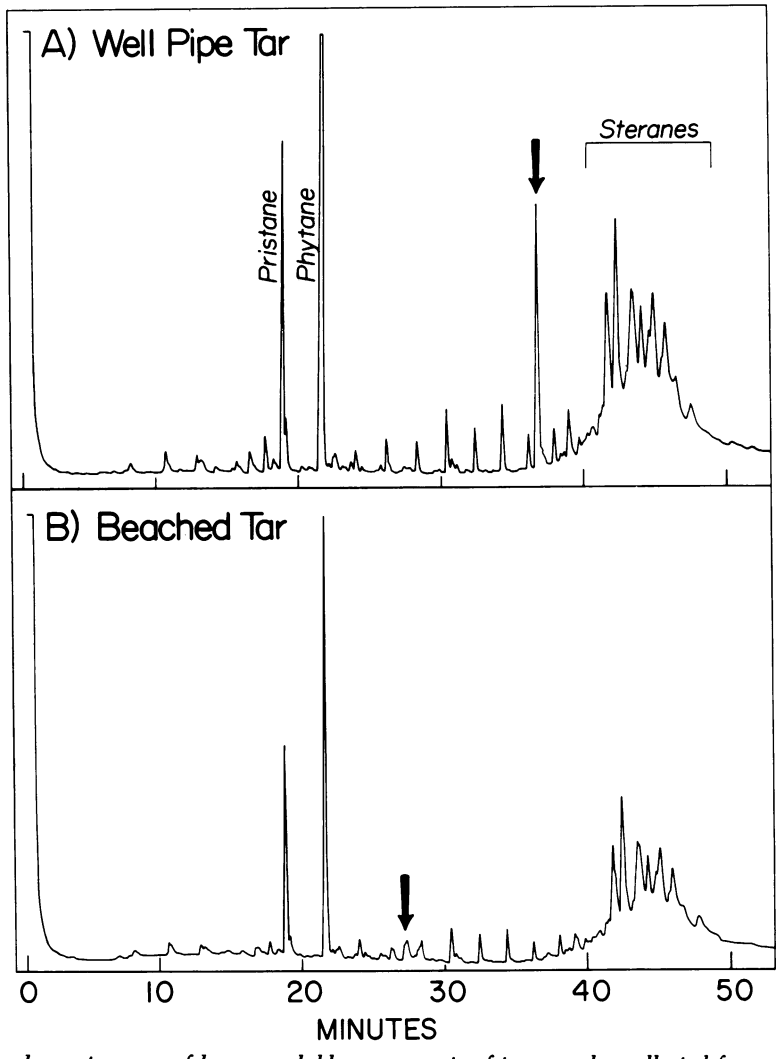

FIG. 5. *Gas chromatograms of hexane-soluble components of tar samples collected from a well pipe (A) and a beach (B) at Rozel Point, Great Salt Lake. See the text for description of chromatographic conditions. Arrows indicate major peaks unique to either sample.*

microorganisms indigenous to chemically and physically similar environments is decreased at increasing salinity. This decrease is apparently not caused by lower levels of dissolved oxygen, since reaeration did not increase oxidation rates. Since halophilic bacteria may be nutritionally fastidious (9, 10), we also tested complex organic compounds for stimulation in highly saline samples. The lack of stimulation by organic compounds supports the argument that the rate limitations were due to salinity and not another variable.

The rates of mineral oil, hexadecane, and glutamate oxidation were all decreased similarly, suggesting that salinity presents a general problem in the metabolism of organic compounds. Results of radiometric assays suggest that, at extreme salinities, the metabolism of glutamate still occurs, albeit at a slow rate, whereas hexadecane oxidation may be completely prevented.

The similar compositions of fresh and beached tar samples from Rozel Point in Great Salt Lake also support the conclusion that hydrocarbons degraded in nonsaline environments are not metabolized in the hypersaline north end of Great Salt Lake, although the length of exposure of the beached tar to weathering is unknown. Since branched and cyclic hydrocarbons are predominant in the tars, extensive biodegradative removal of *n*-alkanes may have occurred after

deposition (2, 3). Alternatively, high levels of pristane and phytane also found in Dead Sea sediments by Nissenbaum et al. (15) may suggest that the tars (or their petroleum precursors) were formed in a hypersaline environment with lipids of halophilic bacteria as a likely source of phytane (11). Since a soil enrichment culture affected removal of phytane relative to other components of the well pipe tar, it seems unlikely that toxic tar components prohibited hydrocarbon metabolism in situ. Although branched-chain alkanes are often noted for their resistance to degradation, microbial metabolism of such hydrocarbons is known (13, 16).

Failure to enrich for microorganisms able to use mineral oil as a sole source of carbon and energy in samples above 20.4% salt also supports the argument that extreme salinity may be a natural barrier to hydrocarbon metabolism.

The reduction of metabolic rate by high salt content of natural hypersaline environments may have significant effects on the cycling of organic compounds in such environments. If, for example, production exceeds decomposition, the sediments of hypersaline lakes may be unusually rich in organic matter. Specific classes of compounds such as hydrocarbons, the metabolism of which may be entirely prevented by extreme salinity, may be unusually abundant in hypersaline lake sediments. This may provide a microbiological basis for the abundance of hydrocarbons in recent sediments of the Dead Sea, and for the general association of petroleum with evaporite deposits (15).

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