Chapter 9

Sodium-Ion Dependence in Marine Gram-Negative Bacteria

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Contents

Introduction

The obligate requirement of a marine bacterium for Na^+ was first demonstrated by Richter (1928). Later studies, undertaken by MacLeod and Onofrey (1956, 1957), led to the postulation that true marine bacteria might be distinguished from terrestrial and freshwater bacteria by having a readily detectable need for Na^+ in the growth medium. It is now known that in gram-negative marine bacteria Na^+ is required by membrane transport proteins, or permeases, to bring substrates into the cell via Na^+ substrate symports (Niven and MacLeod, 1980). This mechanism is shown in Figure 9.1. Na^+ is also required to retain substrate once it is accumulated in the cell, although Li^+ can partially replace Na^+ in this capacity (MacLeod, 1965, 1968).

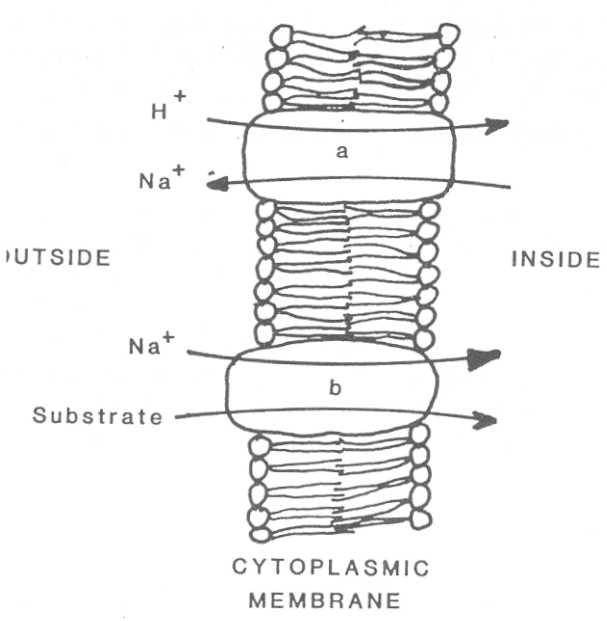

Figure 9.1. A schematic drawing showing that a Na^+ gradient is maintained when Na^+ is extruded by a proton activated antiport, *a*. The Na^+ activates a Na^+-substrate symport when it again enters the cell, *b*. This results in the accumulation of substrate inside the cell.

The taxonomic significance of the Na^+ requirement was firmly established during the 1970s by Paul Baumann and co-workers (Baumann and Baumann, 1981). The ninth edition of *Bergey's Manual in Systematic Bacteriology* (1984) was the first edition of the manual in which it was recognized that gram-negative marine bacteria have a specific growth requirement for Na^+ and can be differentiated from terrestrial and freshwater bacteria on this basis.

Gram-positive bacteria isolated from the sea do not appear to require Na^+ for growth (MacLeod, 1968). However, these organisms make up 5% or less of bacteria isolated from oceans. Gram-negative freshwater bacteria do not require significant amounts of Na^+ for growth and are not found in the open ocean. It is likely that Na^+ confers some selective advantage on marine strains as long as they remain in seawater. Where freshwater enters the ocean there may be a gradual transition between freshwater and marine populations, although the salinity at which this occurs is not known.

In this laboratory exercise, the Na^+ requirement for substrate accumulation by a marine gram-negative bacterium is demonstrated by using a radiolabelled substrate. Similarly, it is demonstrated that a freshwater strain does not require Na^+ for substrate accumulation and that, at higher levels of Na^+, accumulation may even be repressed. From these observations, it can be deduced why each type is specific to its particular habitat.

Student Materials

The materials required by each pair of students is given below. Further information on the preparation of these materials is given in the Notes for the Instructor section and in Appendix A.

1. 50-ml erlenmyer flasks or serum bottles (22)
2. 5 ml suspending salts containing 200 mM LiCl, 50 mM $MgSO_4 \cdot 7H_2O$ and 10 mM KCl. The suspending salts can be made from 200 mM KCl and 50 mM $MgSO_4 \cdot 7H_2O$ if LiCl is not readily available.
3. 100 ml salt solution containing 200 mM $MgSO_4 \cdot 7H_2O$ and 40 mM KCl
4. 25 ml Tris-phosphate buffer pH 7.5 (1.0 M)
5. 100 ml distilled water
6. 75 ml 1.0 M NaCl
7. 4.0 ml α-aminoisobutyric acid (8 KBq per μM or 22μ Ci/μM)
8. 300 ml wash salts (300 mM NaCl, 50 mM $MgSO_4 \cdot 7H_2O$ and 10 mM KCl)
9. Membrane filters, pore size either 0.20 or 0.45 μm (22)
10. A vacuum flask apparatus and vacuum pump
11. Pipets or pipetting devices to measure volumes of 10 ml and less and volumes of 1.0 ml and less

Procedures

1a. This step can be done before the class begins or by the students. Thick cell suspensions of *Pseudomonas fluorescens* (a common terrestrial bacterium) and *Alteromonas communis* (a similar bacterium, but of marine origin) are prepared. This is done, for each of the two strains, by scraping growth from the surface of two petri dishes and suspending the cells in about 1 ml of an artificial seawater solution in which NaCl has been replaced by an equimolar concentration of LiCl.

1b. The amount of thick cell suspension to be added to each flask must be calculated. The amount required is that which will give 100 μg dry weight of cells per ml in 10 ml of a salt solution containing 300 mM NaCl, 50 mM $MgSO_4 \cdot 7H_2O$, 10 mM KCl, and 50 mM Tris-phosphate buffer (pH 7.5). Generally, this amount is obtained when the optical density at 600 nm is 0.40 using a laboratory spectrophotometer. Usually, the amount of thick cell suspension added to each flask is about 25 μ1.

2. Take 11 50-ml erlenmyer flasks and number them A-1 to A-11. The A is for *Alteromonas* sp.

3. Repeat step 2 with a second set of 11 flasks. Number these flasks P-1 to P-11. The P is for *Pseudomonas* sp.

4. To each flask, in both sets, add 2.5 ml of a salt solution containing 200 mM $MgSO_4$ and 40 mM KCl.

5. To each flask, in both sets, add 0.5 ml of 1.0 M Tris-phosphate buffer, pH 7.5.

6a. To each flask, in each set, a stated volume of distilled water must be added. This amount, in addition to the substrate and the amount of NaCl solution to be added in step 7, will bring the final volume in each flask to approximately 10 ml.

6b. The figures that follow give the flask number first followed by the volume of distilled water to be added: 1, 6.8 ml; 2, 6.5 ml; 3, 6.3 ml; 4, 6.1 ml; 5, 5.8 ml; 6, 5.3 ml; 7, 4.8 ml; 8, 4.3 ml; 9, 3.8 ml; 10, 2.8 ml; and 11, 1.8 ml.

7. To each flask, in each set, a stated volume of 1.0 M NaCl must be added. As above, the figures that follow give the flask number first followed by the volume of NaCl to be added: 1, 0 ml; 2, 0.25 ml; 3, 0.5 ml; 4, 0.75 ml; 5, 1.0 ml; 6, 1.5 ml; 7, 2.0 ml; 8, 2.5 ml; 9, 3.0 ml, 10, 4.0 ml; and 11, 5.0 ml. The final concentration of NaCl in the flasks will be as follows: 1, 0 mM; 2, 25 mM; 3, 50 mM; 4, 75 mM; 5, 100 mM; 6, 150 mM; 7, 200 mM; 8, 250 mM; 9, 300 mM; 10, 400 mM; and 11, 500 mM.

8. To each flask ^{14}C-AIB (α-aminoisobutyric acid) must be added. Add 0.16 ml to each flask. Wear gloves and treat the flasks with care from this point on. Report any spills to the laboratory instructor.

9. A volume of thick cell suspension must be added to each flask in a set. This volume will have been determined in step 1b. It is suggested that steps 9 to 12 be completed *before* starting with the second set of flasks.

10. At this step the experiment is timed. After *A. communis* is added to flask A-1, a subsample will be withdrawn 45 minutes later. Add the bacterial suspension to each subsequent flask at a 2-minute intervals until the set is inoculated. This will require 22 minutes.

11. After each flask has been incubated for 45 minutes, withdraw a l-ml sample (this will be done at 2-minute intervals) and filter it by membrane filtration. Wash once with 10 ml of wash salts (300 mM NaCl, 200 mM $MgSO_4 \cdot 7H_2O$, and 10 mM KCl).
12. Remove the filter with forceps and place it in a scintillation vial. Prepare to filter the next sample at the appropriate time.
13. Once a set of filters has been collected, dry them in an oven at 90°C for about 15 minutes or until dry.
14. Steps 10 to 13 must be repeated using *P. fluorescens* as the test organism.
15. Add 10 ml of liquid scintillation fluid to each vial, then cap the vial. Wipe the vials clean with a tissue. The vial caps (not the side of the vial) can be marked A-1, A-2, etc. The student's name or initials should be put on the first and last vials for identification. The samples are now ready for counting.
16. Count the vials for a minimum of 1 minute each in the ^{14}C channel of a liquid scintillation counter. Longer counting times are desirable but may not be practical in class time.

Results and Discussion

Plot counts per minute (cpm) versus Na^+ concentration for the uptake of ^{14}C-AIB by *A. communis* and *P. fluorescens*. A typical set of results is shown in Figure 9.2. After an examination of the results, the students should be able to speculate about the Na^+ concentration at which one organism might have a selective advantage over the other in freshwater, estuarine, and marine environments.

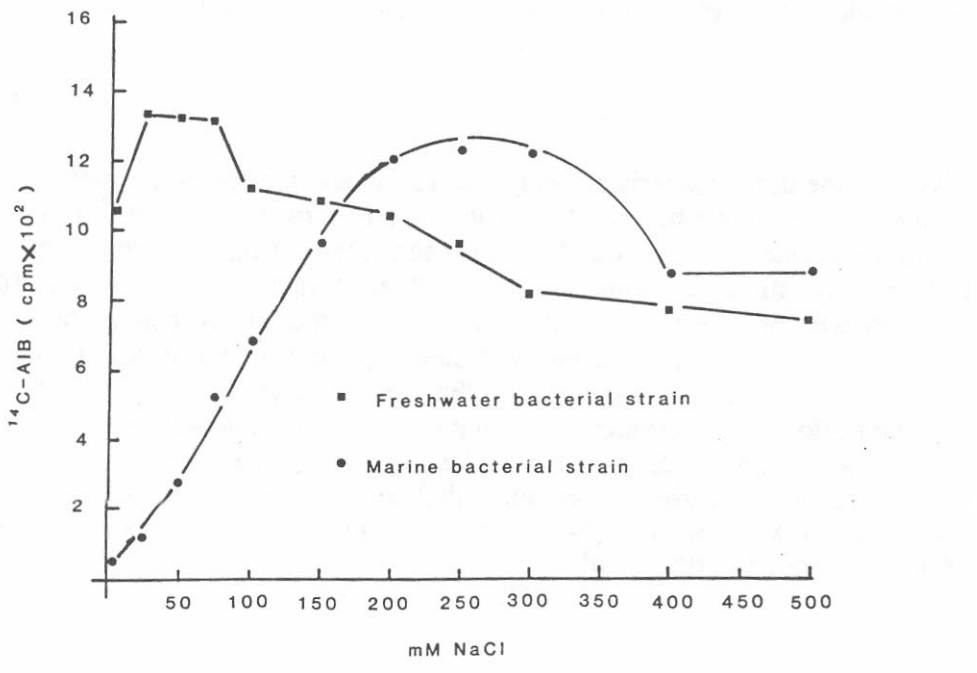

Figure 9.2. Results showing Na^+-dependent substrate accumulation by a marine bacterium, *Alteromonas communis*, and a freshwater bacterium, *Pseudomonas fluorescens*.

Notes for the Instructor

Expertise and Equipment

This exercise is designed for third- or fourth-year students. Students should have completed the equivalent of an introductory microbiology course or another course in which aseptic technique and quantitative manipulations are taught. The organisms used are non-pathogenic and the amount of radioisotope used is minimal. Approximately 5 μCi (200 Kbq) of ^{14}C-labelled substrate is required per pair of students. Safety precautions that minimize risk include using absorbent bench coating, wearing a laboratory coat and disposable latex gloves, and providing pipetting devices so that there is no need to mouth pipette.

A work station is shown in Figure 9.3. This consists of a metal tray with a raised lip. The tray is lined with absorbent paper that is available from scientific supply companies; one side of the paper is absorbent and the other side is lined with plastic. Use the paper with the plastic side down; it is designed to absorb spills, not to protect the tray. If trays are not available, the absorbent paper can be taped directly to the bench top. Any type of filtration apparatus designed for membrane filtration should be adequate. Figure 9.3 shows a manifold with places for three filtration units. A single glass filtration unit, mounted on a stand, would also be adequate. A vacuum flask, used to collect the filtrate, is shown between the manifold and the vacuum pump. Although it is not shown, a second vacuum flask should be present to serve as a trap between the first flask and the vacuum pump.

Some of the materials required for the experiment are shown in Figure 9.4. Good-quality glass pipets will help students to make more accurate measurements of liquids. Pipetting devices such as pi-pumps are required so that the students will not mouth pipette. Variable-volume pipets such as the pipetman are useful, especially for measuring small volumes; a pipetman has a disposable tip. Membrane filters and 50-ml flasks or bottles will be required. Although not shown, it is useful to supply disposable latex gloves. Each student should wear a laboratory coat.

The Experiment

In this experiment, the bacteria are suspended in an artificial seawater medium to which increasing concentrations of NaCl have been added. The range is between 0 and 500 mM Na^+. About 460 mM Na^+ is the natural concentration found in seawater. Depending upon the bacterial strains used, the effect of Na^+ on substrate accumulation is best demonstrated between 0 and 200 mM. Therefore, more Na^+ concentrations are tested in this range than at the higher concentrations. The bacteria are added to the flasks after adding the radiolabelled substrate, which is α-aminoisobutyric acid. This substrate can be transported into the cell by the membrane permease system but is not metabolized by the cell. This allows the measurement of accumulated substrate without the necessity of accounting for substrate lost through metabolism during the course of the experiment. A mixture of radiolabelled and unlabelled substrate is used. The unlabelled substrate is called the carrier. In this experiment, the substrate is a mixture of approximately nine unlabelled molecules for every one radiolabelled molecule of α-aminoisobutyric acid.

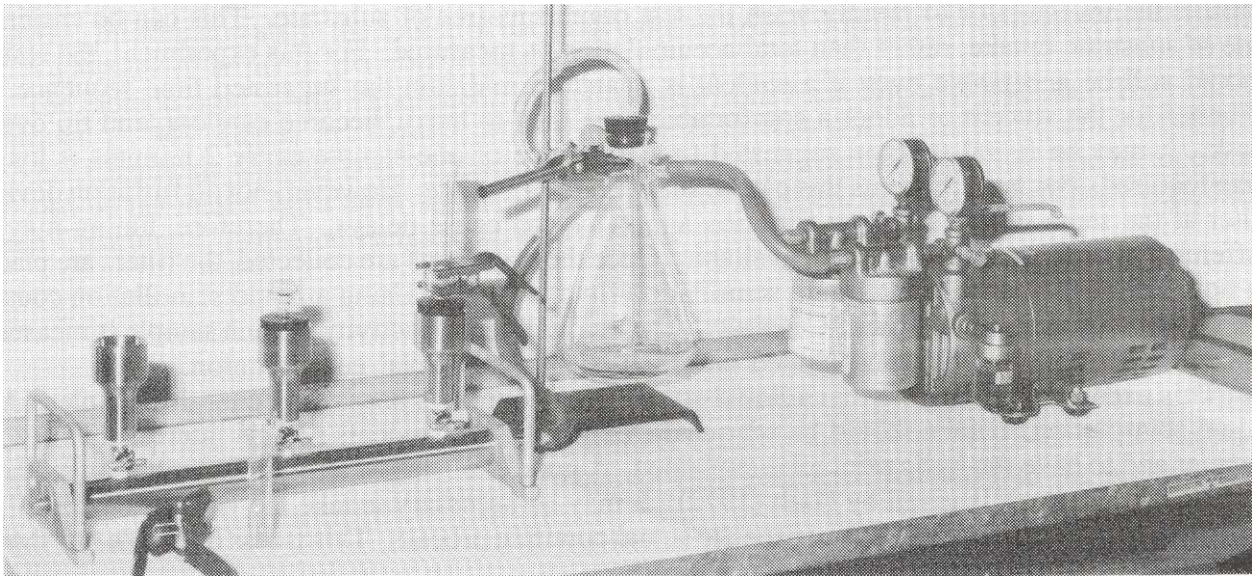

Figure.9.3. A workstation suitable for a pair of student is a vacuum manifold with filter units, a filtrate trap, and a vacuum pump; these are standing on absorbent paper in a tray that has a raised edge.

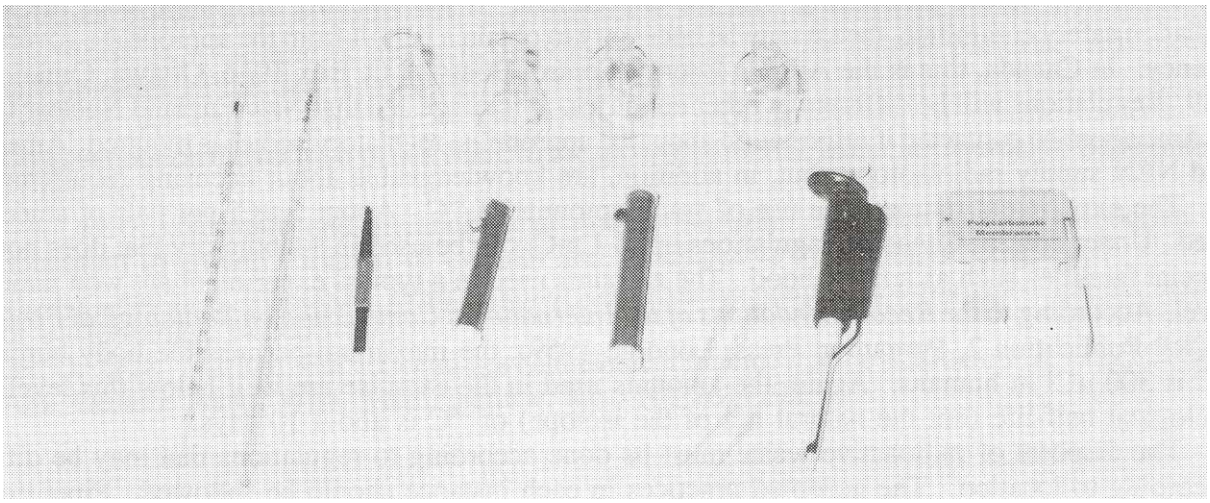

Figure 9.4. Some of the materials required for this exercise: bottles or flasks, membrane filters, pipets, and pipetting devices.

Once the substrate is added to the bacterial suspensions, the flasks are incubated for 45 minutes. For each flask, the organisms in a 1-ml aliquot are collected by membrane filtration. This method permits the investigator to rapidly wash the test organisms free of substrate. This can be critical if rate of substrate uptake, rather than total accumulation, is measured. For this experiment, the results should still be acceptable even if a student is unable to maintain the suggested time sequence. It is better for the student to adopt a comfortable pace than to hurry, become careless, and tip over a flask. It may be found that the suggested time sequence of one sample every 2 minutes is longer than required. For most students this can be shortened to 1 minute. However, not all filtration devices filter at the same rate and a slow filtration apparatus can cause delays. Therefore, timing may be different according to the equipment available. Once the organisms are collected, the filters are placed in liquid scintillation vials and liquid scintillation fluid is added. Using a liquid scintillation counter the samples are then counted for ^{14}C activity. The amount of radioactivity in each sample is measured in counts per minute (cpm) and plotted on graph paper against Na^+ concentration.

This exercise does require that a liquid scintillation counter is nearby to count the samples. Any liquid scintillation counter should be adequate. Generally, the students will be interested to see the counter and to have the basic principles explained. A good explanation of liquid scintillation methods and the process itself is given by Hash (1972). It may be necessary to take the vials to another place for counting and the students may not see the actual counting process. This is less desirable but should not diminish the significance of the experiment.

Liquid scintillation fluids and vials can be purchased from scientific supply companies. Some liquid scintillation fluids are compatible with water. If one of these is used, it will not be necessary to dry the filters before adding the scintillation fluid.

Radiation Control and Safety Aspects

To purchase radioisotopes, it is necessary to have a permit. Most universities will have a radiation safety committee which will regulate the use of radioisotopes. It will be necessary to consult with the committee before proceeding with the experiment. If the teaching institution does not have a radiation safety committee, then it may be necessary to obtain a permit from the appropriate government agency. In Canada, this is the Atomic Energy Control Board, P.O. Box 1046, Ottawa, Ontario K1P 5S9. Regulations will be different in other countries. It is suggested that New England Nuclear (NEN) or Amersham be contacted if information about the appropriate regulatory bodies is required. Amersham and NEN supply radioisotopes but, in addition, are knowledgeable about licensing requirements.

The experiment requires the use of small amounts of ^{14}C. About 5 μCi per pair of students is used. Under current Canadian regulations up to 1 mCi can be used in a laboratory that does not have special facilities such as a fume hood. The amounts used in a typical experiment are well under that level. According to the *Recommendations of the International Commission on Radiological Protection* (ICRP Publication 2, Pergamon Press, London, 1959), the maximum permissible body burden for ^{14}C is 300 μCi in humans. Again, the amounts used in the exercise are well below this level. The biological half-life (not the natural life of the isotope) of ^{14}C is about 10 days.

The disposal of radioactive waste must be done according to regulations that may be different according to location. The accepted practices in each instance should be followed. Pipet tips and any waste that may have isotope on it should be carefully packaged before disposal. This must be done in a fashion such that no one, knowingly or otherwise, can accidently be contaminated, even with the smallest amount of radioisotope.

Other Information

The formulary provided in Appendix A gives methods for preparing the solutions used in this exercise. Information about the source of bacterial cultures and their maintenance is given in Appendix B. A glossary of terms is given in Appendix C.

The isotope used in this experiment was purchased from New England Nuclear. They can be contacted at: 549 Albany St., Boston, MA 02118; or 2453 46th Ave., Lachine, Quebec H8T 3C9; or NEN Chemicals GmbH, D-6072 Dreieich, West Germany, Postfach 401240. The isotope used can also be purchased from other suppliers of radioactive nuclides, such as Amersham Corp., 2636 South Clearbrook, Arlington Heights, IL 60005.

Acknowledgements

Mr. James Ellison of the Biology Department of Memorial University made a significant contribution to this laboratory exercise. He carefully tested a number of bacterial strains and Na^+ and substrate concentrations. His recommendations have been incorporated into the protocol.

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APPENDIX A
Formulary

1. **Substrate** (α-aminoisobutyric acid, A1B) to be used in part 8 of the Procedures section: This requires a mixture of labelled A1B (Solution A) and of carrier or cold A1B (Solution B).

   Solution A: Can be purchased from a supply house such as New England Nuclear (NEN). A 50 μCi vial (0.5 ml) will be sufficient for 10 students working in pairs. I use α-(1-^{14}C)-aminoisobutyric acid (NEC-212). A similar product can be purchased from other supply houses. See Notes for the Instructor section about the licensing requirements for the purchase of radioisotopes.

   Solution B: Unlabelled A1B can be purchased as a powder from supply houses such as Sigma Chemical Co. A 0.01 M solution for A1B can be made by dissolving 0.103 g in 100 ml of water; this is Solution B.

   For class use prepare the substrate as follows:

Solution A	0.1 ml	
Solution B	4.5 ml	
Distilled water		0.4 ml
Total		5.0 ml

2. **Tris-phosphate**, buffer pH 7.5: This is made up as a 1 liter stock solution of Tris (hydroxymethyl) aminomethane. To about 500 ml of H_2O and 121 g of Tris add 1.26 ml of 85% orthophosphoric acid (H_2PO_4). Adjust the pH to 7.5 with concentrated HCl. Bring the final volume to 1 liter and then check the pH once more. Slight adjustment in pH can be made at this time. It may be appropriate to make up less than 1 liter of the buffer. Be sure to allow sufficient volume for adding the HCl to obtain the correct pH. If the Tris is dissolved in too much water at the start then the final volume may be too great.

3. The **suspending salts**, **salts solution** and **wash salts** are straight forward to prepare. Use the concentrations given in the Materials section.

4. **Growth media:** Marine bacteria can be grown on most media that will support the growth of freshwater bacteria except the former have certain special ion requirements. For many marine bacteria, these ion requirements can be met by using a salts solution consisting of 300 mM NaCl, 50 mM Mg $SO_4 \cdot 7H_2O$, and 10 mM KCl. This salts solution will replace distilled water when rehydrating the organic components of the medium. A plating medium made from the salts solution and containing 2 g/liter yeast-extract, 3 g/liter peptone, and 12–15 g/liter agar will give good growth of the organisms used in this exercise. Many types of peptones are available under several trade names; most are suitable, so try that which is available on the shelf before purchasing a specific type. You can order dehydrated marine agar and marine broth from supply houses; they are ready to use after adding distilled water and sterilizing and are the most convenient to use. The terrestrial strain will grow well on a similar medium prepared with distilled water; it will also grow on the marine medium but more slowly than on the freshwater medium.

APPENDIX B
Bacterial Strains

The bacterial strains can be obtained from the American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852. We have used *Pseudomonas fluorescens* ATCC #E-13525 and *Alteromonas communis* ATCC #27118. We have found that the characteristics of Na^+ uptake of marine strains changes somewhat over several years, but any of the Na^+-requiring bacteria described by Baumann and Baumann (1981) should give good results. The bacterial strains suggested grow well and are non-pathogenic.

Maintaining the Cultures

Procedures for cultivating marine bacteria have been described by Baumann and Baumann (1981). The cultures can be maintained on slants. The need for frequent transfer can be greatly reduced by overlaying the slant with sterile mineral oil. Use a hot-air oven (160°C for 3 hours) to sterilize the oil. The overlay is added after growth has been observed on the slant. When it is time to subculture the organisms use a sterile Pasteur pipet to penetrate the oil and then pull up a small amount of growth which can then be used to inoculate fresh broth. Cultures maintained under oil will remain viable for over 1 year.

APPENDIX C
Glossary

Becquerel (Bq): The quantity of a radioactive nuclide that disintegrates at a rate of one disintegration per second (1 dps). *Note*: In SI units the Becquerel is the accepted measure of radioactive nuclide disintegrations. Frequent reference is made to the microcurie because this term is in common usage.

Beta particle: A charged particle emitted from the nucleus of an atom. It has a mass and charge equal to those of an electron.

Biological half-life: This is the time required by the body to rid itself of 50% of the activity of a radioactive nuclide. For ^{14}C this is about 10 days.

Count: Many liquid scintillation counters register an ionizing event as a count. It is usual to report counts per minute (cpm). Depending upon the efficiency of counting, cpm is usually less than the actual number of ionizing events or disintegrations per minute.

Curie: The quantity of a radioactive nuclide that disintegrates at a rate of 3.7×10^{10} atoms per second. Several fractions of the curie are also used. These include the millicurie (mCi) and the microcurie (μCi).

Efficiency: This is a measure of the probability that a count will be recorded when an ionizing event occurs. A counting efficiency of 90% is usual for ^{14}C-labelled compounds.

Half-life: This is the time required for a radioactive substance to lose 50% of its activity to decay. For ^{14}C the natural half-life is over 5,000 years.

Microcurie (μCi): One millionth of a curie (3.7×10^4 disintegrations per second).

Scintillation counter: A machine used to count light emission. The light may be generated by an ionizing radiation interacting with a solvent and a fluor in liquid scintillation fluid.