A NEW METHOD OF DETECTING AND ENUMERATING VIABLE BACTERIA — LIGHT
A NEW METHOD OF DETECTING AND ENUMERATING VIABLE BACTERIA

by

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LITERATURE REVIEW

Enumeration of viable organisms in a natural population has long been a problem for the microbiologist. Many methods have been outlined which detect viable bacteria, however, these procedures are usually limited to a small segment of the population. Growth requirements of micro-organisms vary so greatly that one conventional method, for example plating, cannot be expected to detect all viable cells present in a sample. Waksman (1933) discussed methods of counting bacteria in seawater and stated that the numbers of organisms in seawater and sediment, able to grow by plating, were as many as 1000 times smaller than the numbers observed by direct microscopic examination. Carlucci and Pramer (1957) found as many as 30 to 40% more colonies on poured agar plates as on the surface inoculated plates. They suggested that the difference in count may have resulted from growth of micro-aerophilic or anaerobic organisms beneath the agar surface. These findings disagreed with those of Jannasch and Jones (1959), who did not record any significant difference between counts obtained with agar pour plates and macrocolony counts on membrane filters. Zobell and Conn (1940) and Carlucci and Pramer (1959) found that some marine bacteria were thermosensitive at temperatures prevailing during plating with molten agar. Jannasch and Jones (1959), however, found no difference in colony counts obtained by plating with molten agar as opposed to macrocolony counts on membrane filters.
Kriss (1963) stated that standard media detected not more than 0.1 to 1.0 per cent of the total numbers of micro-organisms in seawater and marine mud observed by direct counting. Jannasch (1958) found during studies on planktonic bacteria by means of a direct membrane filter method, that total counts were difficult to obtain due to the occurrence of forms difficult to distinguish microscopically. MacLeod (1965) in a review, discussed the representation of marine microflora by conventional detection techniques. At present, it would seem that counting marine bacteria by plating or by direct microscopic methods does not give an accurate representation of the viable population.

Problems of enumeration of micro-organisms have not been restricted to the marine environment. Jennison (1937) suggested that the occurrence of clumps of bacteria was the main reason for the differences between direct microscopic and cultural counts of cells in cultures of Escherichia coli. Collins and Kipling (1957) found 6 to 11,000 times as many micro-organisms in fresh water by their direct microscopic method as by plate counts. Strugger (1948) who investigated and developed the acridine orange - fluorescence microscopy method, used it to differentiate between living and dead organisms in the soil. The dye, 3,6- tetra methyl diamin o acridine, will stain the protein of living protoplasm. The method, though not always successful and subject to error, provided a means of detecting living bacteria even though they might not be detected on plating media. Korgaonkor and Ranade (1966) evaluated the acridine orange method as an indicator of viable cells of E. coli. They found the test not always successful and requiring special skill.
An electronic particle counter has been adapted to screen urine specimens as positive or negative for bacteria (Truant et al., 1962). Results of these screenings agreed fairly well with cultural data obtained from plate counts. Mountney and O'Malley (1966) statistically evaluated the electronic and plate counts for estimating bacterial populations. They found that precision of results obtained with the electronic counter were greater than those obtained by plating.

MICROBIOLOGICAL METHODOLOGY WITH RADIOISOTOPES

Many workers have discussed the transportation and concentration of substances inside micro-organisms. Rothstein (1959) stressed the role of the cell membrane in the metabolism of inorganic electrolytes by cells. Solomon (1961) and Kepes and Cohen (1962) investigated transport across cellular membranes. They concluded that many substances can be concentrated inside living cells, by means of energy dependent transport processes, to levels greater than those outside the cells. Drapeau and MacLeod (1965) showed the need for inorganic ions to maintain intracellular solute concentrations in a marine pseudomonad.

The advent of the membrane filter made feasible the sampling of large volumes of fluids for their microbiological content. Goldberg, Baker and Fox (1952) discussed marine sampling with the membrane filter. This filtering technique was found to be of great value to oceanographic research. Atkinson and McFadden (1956) used the membrane filter for the measurement of the incorporation of radioactive
isotopes into cells. Cells which had taken up a labelled substrate were collected on a membrane filter, washed in situ and their radioactivity determined. A single pipetting of labelled cells onto the filter saved time, reduced error and by-passed tedious centrifugation and washing procedures.

**MICROBIOLOGICAL METHODOLOGY WITH RADIOISOTOPES**

**CARBON - $^{14}$ TRACER TECHNIQUES**

Methods by which bacteria have been induced to incorporate radioactive substrates in order to study metabolic pathways, have been demonstrated by Cowie et al. (1950, 1951, 1952). The advantages of tracer techniques include increased analytical sensitivity and ease of detection of labelled substances. Levin (1965) in a review, compares classical microbiological detection techniques to those utilizing radioisotopes. Levin, Harrison and Hess (1956) first reported a method of detecting coliforms, utilizing a radioisotope, $^{14}C$. A water sample is inoculated into a broth containing lactose - $^{14}C$. Labelled carbon dioxide evolved by the coliforms is quantitatively collected as barium carbonate and the radioactivity is related to cell numbers. This test was found to be impractical due to the high cost of the labelled lactose. Improvements in this method have been reported by Levin et al. (1957, 1961). Cost of the test was reduced considerably by replacing lactose with formate - $^{14}C$. Disadvantages of these tests are primarily the inability to pre-determine the range of cell concentration in a sample and the standardization of the test. The authors mention possible variation of metabolism rates of "wild type" coliform cultures.
Levin (1965), in his review, discusses the elements vital to life and the possibilities of using their isotopes for counting microorganisms. Sulphur-35 labelled cysteine has been used to count cells of Serratia marcescens. Ten thousand cells were detected in three minutes by collecting the evolved hydrogen sulphide. This method, however, was restricted to organisms giving off hydrogen sulphide. The radioactive isotope of hydrogen, tritium, has a very low average energy, making counting disintegrations difficult. Phosphorus-32 was discounted as a possibility for counting micro-organisms, since it has a short half-life and also was not metabolized to a gas.

**PHOSPHORUS - 32 TRACER TECHNIQUES**

Phosphorus is an element required by all living cells. Many studies have been conducted on the mechanism of adsorption and utilization of phosphorus by plant cells, rickettsiae, phytoplankton, yeasts and bacteria. Jeener and Brachet (1944) added inorganic phosphate to phosphorus starved yeast cells and found a massive accumulation of a basophilic substance in the cells. This substance was identified by Waime (1948) as polyphosphate (PP). Widra (1959) studied the metachromatic granules of micro-organisms and pictured the volutin granules as PP, ribonucleic acid, lipid, protein and magnesium complexes. The enzyme which controls the formation of PP, polyphosphate kinase, was first detected by Yoshida and Yamataka (1953). Kornberg, Kornberg and Simms (1956) purified the enzyme and found that it required magnesium for activity. Smith, Wilkinson and Duguid (1954) found that volutin granules were phosphorus complexes, requiring for their synthesis, a source of energy, phosphate, potassium and magnesium.
Polyphosphate compounds were not found in cultures starved of carbon, potassium or phosphate. Production of PP was found to be inhibited by sodium azide or 2,4-dinitrophenol. Sodium azide inhibits certain enzymes involved in phosphorylation and cell respiration. Dinitrophenol was believed to interfere with glucose metabolism by interfering with the formation of energy rich phosphate bonds. Factors affecting the accumulation and disappearance of PP in the cells of Corynebacterium diptheriae were studied by Sull, Mudd and Davis (1957). Accumulation of PP in resting cells was favored by substrates such as malate but was inhibited by glucose.

The radioactive isotope of phosphorus, $^{32}$P, a beta ray emitter, has been used extensively in phosphate metabolism studies. Mudd, Yoshida and Kake (1953) studied the incorporation of $^{32}$P into the resting cells of Mycobacterium spp. The cells were incubated for 24 hours in a phosphate accumulation medium containing KCl, malate or glucose, $^{32}$P and Tris (hydroxy methyl) amino methane buffer. A major portion of the tracer was found in the acid insoluble PP fraction. Mallin and Kaplan (1959), investigated the uptake of $^{32}$P as inorganic phosphate, by resting cells of the anaerobe, Clostridium perfringens and found that $^{32}$P incorporation into intact cells occurred only in the presence of glucose. Contrary to previous studies, 2,4-dinitrophenol was found to enhance the incorporation of the tracer. Kaltwasser and Schegel (1959) suggested that the PP cycle in bacterial cells was involved in the control of the inorganic phosphate level in the cells. It was noted that the PP cell content was a function of the cellular phosphate rather than of the
energy supply and that PP accumulation was associated with inhibitory nutritional conditions. Goodman and Rothstein (1957) and Langen, Liss and Lohmann (1962) found P rapidly incorporated into resting yeast cells in the presence of glucose.

Harold and Harold (1963) studied mutants of *Aerobacter aerogenes*, blocked in the accumulation of PP and found that potassium, magnesium, phosphate and an energy source were required for the accumulation of the PP. Robson (1964) labelled a strain of *Staphylococcus aureus* with P and found that best labelling was achieved when the organisms were grown in a broth containing 33 micrograms of orthophosphate per liter. Maximum labelling occurred with organisms previously supplied with sufficient cold phosphate. Organisms previously starved of phosphate, took up the tracer but quickly exchanged it with the cold phosphate in their cells. Myers and McGready (1964) incorporated P into the cells of *Serratia marcescens* by growing the organism in the presence of labelled orthophosphate. They found that P assimilation was influenced by time of incubation, temperature and nutrients present in the culture medium. One per cent glucose was found to stimulate tracer uptake by 34 per cent. Harold (1964) stated that the accumulation of PP in organisms is generally associated with nutritional conditions unfavorable to growth.

Steveninck and Booij (1964) and Steveninck and Rothstein (1965) studied the role of PP in yeast cells and suggested that PP might serve to phosphorylate a carrier for the active transport of glucose.
Harold et al. (1965) found during a study of *Streptococcus faecalis*, that the characteristics of phosphate uptake were not affected by cell wall removal and therefore, assumed the access system to be located in the cell membrane. They suggested a relationship between phosphate uptake and the steady state level of glycolytic intermediates. Szymona and Szumilo (1966) suggested that PP is utilized for the direct phosphorylation of hexoses.

Radioactive phosphorus and tracer labelled cells have found much use in the study of movements of organisms and of components of the phosphorus cycle in natural environments. Rice (1953) investigated phosphorus exchange between the culture medium and marine planktonic algae. He found that phosphorus was exchanged by cells that were not photosynthesizing or dividing and also by actively metabolizing cells. Buckland (1950) used bacterial spores labelled with $^{32}$P to study the respiratory retention of aerosols. Tracer studies on the phosphorus metabolism of *Escherichia coli* were carried out by Labaw, Mosley and Wyckoff (1950). These workers used the phosphate labelled *E. coli* cells to study the phosphate metabolism of a bacteriophage. Stonier (1956) labelled crown gall bacteria with $^{32}$P. The technique of autoradiography was used to locate the labelled cells in the infected plants. Rigler (1956), Lebdeva (1957) and Gak (1962) studied the accumulation of radioactive phosphorus by plankton and other components of lakes and reservoirs.
Hoyers and Pickens (1962) prepared labelled *Coxiella burnetii*. Tracer levels of 0.05 to 0.125 millicuries were used to label the organisms.

Several studies have been carried out with algae. Simonis and Urbach (1956) studied the uptake of labelled phosphate by the unicellular alga, *Ankistrodesmus braunii*. They found that the incorporation of the tracer was stimulated by sodium, more in the light than in the dark. They concluded that sodium affects the formation of energy rich phosphate bonds. Baker and Schmidt (1964) studied the intracellular distribution of phosphate in the cells of *Chlorella pyrenoidosa* during synchronous growth and found that the concentration of PP fluctuates with a maximum being reached just prior to nuclear division. Phosphorus accumulation in phosphate deficient *Scenedesmus* spp. was found to be dependent upon light intensity (Kylin, 1966). Phosphorus accumulation in plants has been studied, particularly in connection with fertilizer utilization. A study into the mechanism of transport of phosphorus into barley shoots has been carried out by Loughman (1966), who found that transport requires prior incorporation of the phosphate into organic compounds. The accumulation of phosphorus compounds by fungi has been studied. James and Casida (1964) and Siegenthaler and Goldstein (1967) found a net uptake of tracer stimulated by sodium chloride and magnesium chloride. A profound effect of inorganic phosphate upon the
utilization of glucose by red blood cells has been noted by Rose, Warms and O'Connell (1964).
MATERIALS AND METHODS

CULTURES

Organisms used in this study included one terrestrial and seven marine bacterial species. These cultures were:

(1) *Aerobacter aerogenes*, Macdonald College Culture Collection # 112.
(2) *Pseudomonas* species strain B-16. This organism was originally isolated by MacLeod and Onofrey (1954) from a marine clam on the Pacific Coast of Canada. It has been classified as a *Pseudomonas* species Type IV by the Torrey Research Group and is lodged in the National Collection of Marine Bacteria, Torrey Research Station, Aberdeen, Scotland, as NCMB 19. It has been entered in American Type Culture Collection where it has been given the ATCC No. of 19855.
(3) *Pseudomonas* B-10, NCMB 370.
(4) *Cytophaga* B-9, NCMB 292, ATCC No. 19854.
(5) *Vibrio* MB-22. The original culture of this organism was received from Dr. D. Pratt, formerly of the University of Florida.
(6) *Leucothrix mucor*. The original culture of this organism was obtained through the courtesy of Dr. Thomas D. Brook, University of Indiana, Bloomington, Indiana.
(7) *Gaffkya homari*, ATCC No. 10400.
(8) *Photobacterium fisheri*. This organism was obtained from Dr. W.D. McElroy of Johns Hopkins University.
All cultures were carried on agar slants and were transferred bi-weekly. All marine organisms with the exception of G. homari, were inoculated on slants of Trypticase Seawater Agar, Table I, incubated for 18 to 24 hours at 25°C and then held at 4°C until required. Gaffkya homari was inoculated onto slants of Brain Heart Infusion Agar (Difco), incubated for 18 to 24 hours at 30°C and held at 4°C until required.

Aerobacter aerogenes was inoculated onto slants of Trypticase Soy Agar (Baltimore Biological Laboratories), incubated for 18 to 24 hours at 30°C and then held at 4°C until required.

**GROWTH AND HARVESTING OF CELLS**

One loopful of cells from a slant was used to inoculate 200 ml. of broth. A. aerogenes was grown in Trypticase Soy Broth (BBL); G. homari, a gram - positive marine organism, was grown in Difco Brain Heart Infusion Broth while all the other marine bacteria were grown in Trypticase Seawater Broth (Table I). The cells were grown on a rotary shaker, A. aerogenes for 24 hours at 30°C, G. homari for 24 hours at 30°C, Pseudomonas B-16 and Ph. fisheri for 16 hours at 25°C, Pseudomonas B-10, L. mucor and Vibrio MB-22 for 18 hours at 25°C and Cytophaga B-9 for 24 hours at 25°C. The cells were harvested by centrifugation at 10,000 RPM and 4°C and were washed three times by centrifugation from and resuspension in filter sterilized washing fluid. Washing fluid in the case of terrestrial organisms, was glass distilled water and in the case of marine organisms, was seawater.
TABLE I

The composition of the media used to carry and cultivate the marine organisms.

**TRYPICASE SEAWATER BROTH**

- Trypticase (BBL) ............. 10 gm.
- FeSO\(_4\) (NH\(_4\)) SO\(_4\) \(\cdot\) 6 H\(_2\) O ............. 0.1 mM.
- Aged seawater ............. 1000 ml.

**TRYPICASE SEAWATER AGAR**

- Trypticase Seawater Broth ............. 1000 ml.
- Agar ............. 15 gm.

The above media were sterilized by autoclaving at 15 pounds per square inch pressure for 15 minutes.
The suspension of cells was adjusted to a level of approximately \(1 \times 10^8\) cells per ml. by referring the turbidity of an aliquot of the suspension to a previously calibrated curve relating numbers of cells to turbidity. Serial dilutions of the suspensions were made with the respective diluents to give the desired cell concentrations for the experiments.

**PHOSPHORUS \(^{32}\) SOURCE**

Carrier - free radioactive phosphorus as \(\text{H}_3\text{PO}_4\), pH less than 1, was obtained from the Atomic Energy Commission of Canada. The volume received was neutralized by diluting with 0.05M Tris (hydroxy methyl) amino methane buffer, pH 7.2. Since this radioactive isotope has a half-life of 14.3 days, a computation of the actual amount of radioactivity present in the source was made before each experiment. An aliquot of the \(\text{P}^{32}\) source was then diluted with sufficient Tris buffer, filter sterilized glass distilled water or seawater as desired, to give 10 microcuries \(\text{P}^{32}\) per ml. This concentrate was filter sterilized by passing the solution through a sterile HA Millipore filter (0.45 micron pore size). Filtration was necessary, not only for sterilization, but also to remove radioactive particulate matter which, if allowed to remain, caused the counts on the experimental filters to be extremely erratic.
GLASSWARE

All glassware was scrupulously cleaned by washing first with detergent, then by soaking 24 hours in potassium dichromate - sulphuric acid cleaning solution. Glassware was then rinsed six times in glass distilled water. All glassware with the exception of pipettes, was sterilized by autoclaving for 15 minutes at 15 pounds per square inch pressure. Pipettes were sterilized by dry heat, in clean metal cylinders.

FILTERS

Filters used in this study were usually Millipore HA type (0.45 micron pore size). Equally effective and used where noted were the GA - 6 Gelman Metricel filters. These filters were wrapped in paper and were sterilized at 15 psi for 15 minutes.

INCUBATION MEDIUM AND LABELLING PROCEDURE

The composition of the incubation medium used with _A. aerogenes_ is shown in Table II. The KCl, MgSO₄ and glucose were dissolved in sufficient glass distilled water to give 10/8 of the final concentration in the incubation medium. This solution was sterilized by filtration. To 8 ml. of this solution in a sterile 25 ml. erlenmeyer flask, was added 1 ml. of P solution (10 microcuries/ml.), and 1 ml. of an appropriately diluted suspension of _A. aerogenes_.

The incubation medium for all marine organisms was filter sterilized seawater. To 8 ml. of seawater in a sterile flask was added 1 ml. P solution and 1 ml. of an appropriately diluted
### COMPOSITION OF THE INCUBATION MEDIUM

<table>
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<tr>
<td>KCl</td>
<td>0.005 Molar</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.002 Molar</td>
</tr>
<tr>
<td>Glucose</td>
<td>10 mg./ml.</td>
</tr>
<tr>
<td>$^{32}$P (in 0.05 M Tris buffer)</td>
<td>1 microcurie per ml.</td>
</tr>
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</table>
suspension of marine bacteria.

A blank without cells was prepared by substituting 1 ml of sterile diluent for 1 ml of cell suspension. A blank with cells was prepared by replacing 1 ml of cell suspension with 1 ml of cell suspension boiled for five minutes.

All flasks were incubated on a rotary water bath shaker for a given time at a known temperature, according to the experiment.

DETECTION OF P-32 RETAINED BY THE CELLS

A one ml. portion of the incubation mixture was filtered through a sterile 25 mm. HA Millipore filter held in place by a Pyrex Microanalysis filter apparatus. The cells on the filter were washed by drawing through 5 ml. filter sterilized glass distilled water or seawater as required. Each incubation mixture was sampled in quintuplicate unless otherwise noted. Blanks without cells or with boiled cells were treated in the same manner. Between samplings, the funnel of the filter apparatus was immersed in 70% ethanol for one minute and then was rinsed with sterile glass distilled water.

Each filter was placed on a clean planchet and air dried. The radioactivity retained on the filters both in the case of the cell samples and the blanks, was determined using a thin end window, Geiger-Muller tube attached to a Picker Scaler. The scaler was preset to record 4000 counts and the time to accumulate this number of counts in the case of each sample was recorded. Results, unless otherwise indicated, were recorded as counts per minute per ml of incubation mixture filtered.
All deviations recorded are average deviations from the mean.

**ENUMERATION OF VIABLE CELLS IN THE INCUBATION MEDIUM**

The numbers of viable cells in each incubation mixture were determined by the standard drop plate technique using, in the case of *A. aerogenes*, Trypticase Soy Agar, with *G. homari*, Brain Heart Infusion Agar and with marine organisms, Trypticase Seawater Agar. Plates were incubated at the appropriate temperatures until the colonies were sufficiently large to count readily. To obtain a count valid to within a coefficient of variation of 7%, 400 colonies were counted in the case of each sample. In order to insure the achievement of a maximum count, particularly in the case of a mixed population, plates were held for an additional period of time at the incubation temperature and were recounted.

To confirm the sterility of filter sterilized solutions, experimental blanks without cells and blanks containing boiled or formalized cells, aliquots of these solutions were plated and incubated under the same conditions as were used to enumerate the organisms under consideration.

**DETERMINATION OF THE TOTAL CELL COUNT IN THE INCUBATION MEDIUM**

The number of bacterial cells in an incubation mixture was determined by the standard direct microscopic count. After the determination of the radioactivity retained by the cells on a filter, the filter with cells was returned to the filter holder and 5 ml. of a solution of Loeffler's Methylene Blue (1:5) (Table III) was added. After 20 minutes, the excess dye solution was drawn through the filter by suction.
<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
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<tbody>
<tr>
<td>Methylene Blue</td>
<td>0.30 gm.</td>
</tr>
<tr>
<td>Ethanol (95%)</td>
<td>30.0 cc.</td>
</tr>
<tr>
<td>Potassium hydroxide (0.01%)</td>
<td>100.0 cc.</td>
</tr>
</tbody>
</table>

The above mixture is diluted 1:5 with glass distilled water.
The dye solution is filter sterilized by passing the solution through a 0.45 micron pore size membrane filter.
The filter was washed free of dye by drawing through the filter, 20 ml. of glass distilled water or until the filtrate became colorless. The filters were then dried for at least two hours and were cleared with immersion oil. A one square centimeter portion of the cleared filter was mounted in a drop of immersion oil on a clean glass slide and covered with a #1 coverslip. The filter was examined microscopically with the oil immersion lens. Fifty microscopic fields of known dimensions were counted and the counts were reported as cells per ml. of incubation mixture filtered. Clumps of organisms or questionable stained objects were not counted but were noted for the sample concerned.

THE DETERMINATION OF THE DRY WEIGHT OF A CELL SUSPENSION IN THE PRESENCE OF SALTS

The dry weights of cell suspensions in the presence of seawater were determined by a method which took into consideration, the interference of the weight of salts in the suspending fluid (Buckmire, personal communication).

Duplicate 1 ml. samples of the cell suspension were taken for dry weight determination. These samples were placed in separate 5 ml. preweighed beakers and dried at 80°C overnight. They were placed in a desiccator and were dried to constant weight. Two 1 ml. samples of the suspending fluid, filter sterilized seawater, were taken and the dry weights were similarly determined. Five ml. of the cell suspension was centrifuged at 50,000 times gravity for
forty minutes. The supernate was discarded and the cells were brought to volume with double strength seawater, prepared by evaporating seawater to one half the original volume. The cells were resuspended and duplicate 1 ml. samples were taken for dry weight determinations. The remainder of the cell suspension was centrifuged at 50,000 times gravity for 40 minutes. Duplicate 1 ml. samples were taken of the supernate and dry weights were determined. The duplicate results in all cases were averaged. The dry weight of the cells in suspension was determined by the following formulae.

\[ Y = n (1 - v ) + A \]  
(1)

\[ X = w (1 - v ) + A \]  
(2)

where \( v \) represented the volume (ml.) of the cells in 1 ml. of the original suspension. Then \( 1-v \) represented the volume of the salt solution (seawater). Let \( n \) represent the dry weight of 1 ml. of the suspending fluid (seawater), \( w \) the dry weight of 1 ml. of twice strength suspending fluid, \( Y \) the total weight of 1 ml. of seawater plus cells, \( X \) the total weight of 1 ml. of the twice strength seawater plus cells and \( A \) the actual dry weight of cells per ml. of cell suspension. From formulae (1) and (2) the following simultaneous equations could be set up.

From (1) \[ A = Y - (n) (1 - v) \]  
(3)

From (2) \[ A = X - (w) (1 - v) \]  
(4)

Then \[ Y - (n) (1-v) = X - (w) (1-v) \]  
(5)

Solving for \( v \) from (5).

\[ v = \frac{Y-X + (w-n)}{(w-n)} \]  
(6)

Knowing \( X, Y, n \) and \( w \), equation (6) was solved for \( v \).

The calculated value for \( v \) was used in either equation (3) or (4) to solve for \( A \).
SEAWATER SAMPLES

The seawater samples for this study were examined in two groups. The first group of samples were taken June 1, 1966, from the open ocean, 2 miles off Terence Bay, Nova Scotia, during an incoming tide. The samples were collected aseptically in one liter bottles, from the water at a depth of three feet, by uncorking sterile bottles at this depth and allowing them to fill. These samples were tested within two hours of collection, during which time they were kept at 4°C.

The second group of samples was received July 26, 1966, by Air Express, from the Technological Station of the Fisheries Research Board, Halifax, Nova Scotia. These samples were collected in a manner similar to that previously described and were tested within 12 hours of collection. During shipment, these samples were kept in the cold.
RESULTS

STUDIES CONDUCTED WITH AEROBACTER AEROGENES

EFFECT OF COMPOSITION OF THE INCUBATION MEDIUM ON THE EXTENT OF LABELLING OF CELLS

A series of experiments was carried out with Aerobacter aerogenes to determine the incubation medium that would lead to optimum uptake of $\text{P}^{32}$ by the cells. Various compounds were added to suspensions of cells in distilled water to determine their effect on the rate and extent of labelling of the cells by $\text{P}^{32}$. Compounds were chosen as a result of consideration of those substances which might be expected to be most concerned with the stimulation of phosphate uptake. Myers and McCready (1964), during a study with Serratia marcescens, found that one per cent glucose stimulated $\text{P}^{32}$ uptake by 34 per cent. Uptake of labelled phosphate by resting cells of Clostridium perfringens was found to occur only in the presence of an oxidizable substrate such as glucose or ethanol (Mallin and Kaplan, 1959). Groucher, Sarachek and Kocholaty (1955) showed that Mg++ was important for the maintenance of respiratory activity in Azotobacter spp. Solomon (1961) suggested that potassium was linked to phosphate metabolism in Escherichia coli. The effect of K+, Mg++, and glucose alone and in combination, on the uptake of labelled phosphate by cells of Aerobacter aerogenes was therefore examined.
As seen in Table IV, KCl and to a lesser extent, glucose, tested separately, stimulated P uptake. Magnesium sulphate depressed uptake of the tracer. When magnesium sulphate was tested in the presence of KCl and glucose, however, the three components in combination stimulated uptake more than any one of the compounds tested alone. Tris buffer, tested at a level of 0.05 M, pH 7.2, as well as cysteine at 10 M, were both found to be inhibitory.

The optimum levels of incubation medium components were determined by omitting one of the components and testing its effect at different concentrations in the presence of the other two. Figure 1 shows the effect of KCl concentration on P uptake by A. aerogenes. A clearly defined optimum level of 0.005 M KCl was noted. With this level of KCl in the medium, and glucose at 10 mg. per ml., the optimum level of magnesium sulphate was found to be 0.002 M (Figure 2). Glucose was found to be most effective at 10 mg. per ml. (Figure 3).

TIME REQUIRED FOR LABELLING CELLS

With the optimal levels of incubation medium components as determined previously, the time required for maximal uptake of P was found to be 60 minutes (Figure 4). This experiment was conducted with approximately 500 cells per ml. When the number of cells was increased to 6500 per ml., the time for maximal labelling was increased to 90 minutes. Since most of the experiments were conducted with 500 cells per ml. or less, the incubation period selected for further study was one hour.
TABLE IV

Effect of the various components of the incubation medium, tested alone and in combination, on the uptake of P by cells of *Aerobacter aerogenes.*

<table>
<thead>
<tr>
<th>Additions to Glass Distilled Water</th>
<th>Radioactivity on filter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No cells</td>
</tr>
<tr>
<td>None</td>
<td>2560 ± 51</td>
</tr>
<tr>
<td>KCl (0.005 M)</td>
<td>2890 ± 144</td>
</tr>
<tr>
<td>MgSO₄ (0.002 M)</td>
<td>2701 ± 81</td>
</tr>
<tr>
<td>Glucose (10 mg/ml)</td>
<td>3035 ± 136</td>
</tr>
<tr>
<td>KCl + MgSO₄</td>
<td>2790 ± 68</td>
</tr>
<tr>
<td>KCl + Glucose</td>
<td>2666 ± 79</td>
</tr>
<tr>
<td>MgSO₄ + Glucose</td>
<td>2956 ± 144</td>
</tr>
<tr>
<td>KCl + MgSO₄ + Glucose</td>
<td>2857 ± 71</td>
</tr>
</tbody>
</table>

Suspensions contained an average of 500 cells per milliliter as determined by drop plate count.
FIGURE 1

The effect of KCl concentration on the uptake of $^{32}$P by cells of *Aerobacter aerogenes* in suspension. The incubation medium contained MgSO$_4$, 0.002 Molar, glucose at 10 mg. per ml. and 1 microcurie per ml. of $^{32}$P in Tris buffer, 0.05 Molar, pH 7.2. Cells were suspended at a level of approximately 345 cells per ml.

FIGURE 2

The effect of MgSO$_4$ concentration on the uptake of $^{32}$P by cells of *Aerobacter aerogenes* in suspension. The incubation medium contained KCl 0.005 Molar, glucose at 10 mg. per ml. and one microcurie per ml. of $^{32}$P in Tris buffer, 0.05 Molar, pH 7.2. Cells were suspended at a level of approximately 345 cells per ml.
The effect of glucose concentration upon the uptake of $^{32}$P by a cell suspension of *Aerobacter aerogenes*. The incubation medium contained 0.005 M KCl, 0.002 M MgSO$_4$ and 1 microcurie per ml. of $^{32}$P in 0.05 M Tris buffer, pH 7.2.

Cells suspended at a level of approximately 325 cells per ml.
FIGURE 3

COUNTS PER MINUTE PER CELL

GLUCOSE (mg./ml.)
FIGURE 4
FIGURE 4

The effect of incubation time on the extent of labelling of cells of *Aerobacter aerogenes* in suspension. Cells suspended at a level of approximately 500 cells per ml.

1) Counts per minute reported as averages of quintuplicate samples.
EFFECT OF TEMPERATURE

Preliminary experiments had established that the most rapid labelling occurred when the incubation temperature was 37°C. Since many bacterial species cannot survive at 37°C, it was of interest to know whether appreciable labelling of the cells could occur at lower temperatures. As seen in Table V, cells of A. aerogenes became as extensively labelled at 20°C as at 37°C, however, at the lower temperature, 100 minutes rather than 60 minutes was required for maximum labelling.

EFFECT OF P-32 CONCENTRATION

Various levels of P-32 were tested for their effect on the optimal uptake of the tracer by the cells. The maximal uptake of P-32 by the cells (Figure 5), occurred with one microcurie per ml. of incubation medium. Both above and below this level, the counts per minute per cell were lower.

EFFECT OF WASHING SOLUTION

Since the composition of the washing solution is known to affect the extent of retention of intracellular solute in the case of marine bacteria (Drapeau and MacLeod, 1965), the effect of washing on the retention of P-32 by labelled cells was examined. With marine bacteria, conditions optimal for the uptake of substrates were also optimal for the prevention of their release. However, with Aerobacter aerogenes, washing the cells with increasing volumes of incubation
TABLE V

Effect of incubation temperature on the time required for maximal uptake of $^{32}$P by *Aerobacter aerogenes*.

<table>
<thead>
<tr>
<th>Incubation time (min.)</th>
<th>Incubation temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20$^\circ$C</td>
</tr>
<tr>
<td>0</td>
<td>2582 ± 175</td>
</tr>
<tr>
<td>60</td>
<td>3547 ± 571</td>
</tr>
<tr>
<td>80</td>
<td>3893 ± 541</td>
</tr>
<tr>
<td>100</td>
<td>6026 ± 533</td>
</tr>
<tr>
<td>115</td>
<td>4673 ± 395</td>
</tr>
<tr>
<td>150</td>
<td>4289 ± 145</td>
</tr>
</tbody>
</table>

Suspensions contained an average of 900 cells per milliliter as determined by drop plate counts.
FIGURE 5

The effect of the level of $^{32}$P in the incubation medium on the extent of labelling of cells of *Aerobacter aerogenes* in suspension. Cells suspended at a level of approximately 450 cells per ml.
FIGURE 5

COUNTS PER MINUTE PER CELL

MICROCURIES $^{32}$P PER ML.
medium caused progressively increasing losses of \( ^{32} \text{P} \) (Table VI). Washing with distilled water, however, did not remove the isotope. It should be noted that at least part of the decrease in radioactivity caused by washing with incubation medium is due to the removal of \( ^{32} \text{P} \) bound to the filter.

**LIMITS OF DETECTABILITY OF CELLS BY MEANS OF \( ^{32} \text{P} \) LABELLING**

The range of cell numbers detectable by the \( ^{32} \text{P} \) labelling technique was determined by observing the increase in \( ^{32} \text{P} \) activity on the filters as the numbers of cells in suspension in the incubation medium was increased. The radioactive count increased sharply as the number of cells increased from 0 to about 7000 cells per ml. and then dropped off (Figure 6). The lowest concentration of cells tested was 23 cells per ml. and the results in Table VII show that this number of cells could be detected with 95% confidence.

**EFFECT OF UNLABELLED PHOSPHORUS**

From the results in Figure 6 it can be calculated that the counts per minute per cell decreased approximately logarithmically as the numbers of cells in suspension increased. Since there was a limit to the amount of \( ^{32} \text{P} \) which could be added to the incubation medium (Figure 5), the effect of adding unlabelled as well as labelled phosphate to the incubation medium was determined. It was calculated that the amount of phosphorus added as \( ^{32} \text{P} \) was \( 3.49 \times 10^{-8} \) g/ml.

Unlabelled phosphate as \( (\text{NH}_4)^+ \) \( \text{HPO}_4 \) was added to the incubation medium at 10 and 100 times this level. The results (Table VIII) show
TABLE VI

Effect of type and volume of washing fluid on the retention of $^{32}\text{P}$ by labelled cells of *Aerobacter aerogenes*.

<table>
<thead>
<tr>
<th>Volume (ml)</th>
<th>Washing fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glass distilled water</td>
</tr>
<tr>
<td>Filter + cells</td>
<td>Blank</td>
</tr>
<tr>
<td>5</td>
<td>5012 ± 184</td>
</tr>
<tr>
<td>10</td>
<td>4199 ± 266</td>
</tr>
<tr>
<td>15</td>
<td>4910 ± 554</td>
</tr>
<tr>
<td>20</td>
<td>5137 ± 257</td>
</tr>
</tbody>
</table>

Results are expressed as counts per minute.

The incubation medium used for washing was prepared without adding $^{32}\text{P}$.

Suspensions contained an average of 800 cells per ml. as determined by drop plate count.
The effect of cell concentration on the radioactivity retained by the filters after incubating cell suspensions of \textit{Aerobacter aerogenes} in the presence of $^{32}$P.

1) Counts per minute reported as averages of quintuplicate samples.
2) Cells per milliliter determined by the standard drop plate technique.
FIGURE 6

Counts per Minute vs. Cells per Milliliter
TABLE VII

Statistical analysis of the degree of significance to be attached to the $^{32}$P uptake technique for detecting cells when 23 cells per ml. are present in suspension.

<table>
<thead>
<tr>
<th>Determination</th>
<th>$X_1$ (blank, no cells)</th>
<th>$X_2$ (23 cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Values obtained</td>
<td>3571</td>
<td>4608</td>
</tr>
<tr>
<td></td>
<td>2861</td>
<td>4866</td>
</tr>
<tr>
<td></td>
<td>2826</td>
<td>2838</td>
</tr>
<tr>
<td></td>
<td>2921</td>
<td>4819</td>
</tr>
<tr>
<td></td>
<td>2867</td>
<td>3739</td>
</tr>
<tr>
<td>Mean</td>
<td>3009</td>
<td>4174</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>315.9</td>
<td>872.0</td>
</tr>
</tbody>
</table>

Values reported are counts per minute per ml. of incubation mixture filtered.

Student's t calculated = 2.807. Student's t from tables = 2.306 (t.05).

Therefore, the means are significantly different at the 95% confidence level.
**TABLE VIII**

Effect of unlabelled phosphorus on $^{32}$P uptake by cells of *Aerobacter aerogenes*.

<table>
<thead>
<tr>
<th>Cells per ml.</th>
<th>Unlabelled P added (g/ml)</th>
<th>0</th>
<th>$3.49\times10^{-7}$</th>
<th>$3.49\times10^{-6}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2783 $\pm$ 108</td>
<td>2312 $\pm$ 69</td>
<td>2774 $\pm$ 154</td>
<td></td>
</tr>
<tr>
<td>5.2 $\pm$ 1.1</td>
<td>3565 $\pm$ 245</td>
<td>2781 $\pm$ 162</td>
<td>2182 $\pm$ 252</td>
<td></td>
</tr>
<tr>
<td>620 $\pm$ 13</td>
<td>4629 $\pm$ 230</td>
<td>2528 $\pm$ 200</td>
<td>2643 $\pm$ 208</td>
<td></td>
</tr>
<tr>
<td>61830 $\pm$ 1.1</td>
<td>7561 $\pm$ 225</td>
<td>3392 $\pm$ 334</td>
<td>3418 $\pm$ 170</td>
<td></td>
</tr>
</tbody>
</table>

Unlabelled P added as (NH$_4$)$_2$HPO$_4$.

Results reported as counts per minute.
that even 10 times the level markedly depressed the uptake of P
even though the amount of unlabelled phosphate this represented
was still very small.

**RELATION OF CELL VIABILITY TO P-32 UPTAKE**

To determine whether the uptake of P\(^{32}\) by the cells required
that the cells be viable, the effect of heat, 2,4- dinitrophenol
(DNP), and formaldehyde on the capacity of the cells to take up
the tracer was examined (Table IX). It was evident that the
\(^{32}\) greatest amount of P\(^{32}\) uptake occurred with untreated cells and
the least was obtained in the presence of DNP. In both cases,
the cells could be shown still to be viable on subsequent plating
of the suspensions. Though no viable cells remained after heating
\(^{32}\) or formaldehyde treatment, some uptake of P\(^{32}\) over the corres-
ponding blank was obtained. It was also of interest to note that
the radioactivity was less on filters through which DNP-treated
cell suspensions were filtered than on filters through which the
incubation medium containing no cells was filtered.
The effect of heating, formaldehyde treatment and 2,4-dinitrophenol (DNP) on the capacity of cells of *Aerobacter aerogenes* in suspension to take up P³.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Radioactivity on filter</th>
<th>Viable bacteria per ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>2698 ± 304</td>
<td></td>
</tr>
<tr>
<td>Blank + DNP</td>
<td>2334 ± 744</td>
<td></td>
</tr>
<tr>
<td>Blank + formaldehyde</td>
<td>2638 ± 218</td>
<td></td>
</tr>
<tr>
<td>Cell suspension</td>
<td>5144 ± 536</td>
<td>835</td>
</tr>
<tr>
<td>Cells + DNP</td>
<td>1364 ± 405</td>
<td>789</td>
</tr>
<tr>
<td>Cells + formaldehyde</td>
<td>3418 ± 294</td>
<td>0</td>
</tr>
<tr>
<td>Cells + heat</td>
<td>3860 ± 406</td>
<td>0</td>
</tr>
</tbody>
</table>

DNP concentration, 10⁻³ Molar.

Formaldehyde concentration, 1%.

Heat treatment consisted of heating the cell suspension to 100°C for 5 min.
STUDIES CONDUCTED WITH MARINE BACTERIA

FACTORS AFFECTING THE LABELLING OF MARINE BACTERIA WITH P-32.

Studies on the uptake of P$^{32}$ by cell suspensions of Aerobacter aerogenes had shown that the composition of the incubation medium influenced the rate and extent of labelled phosphate uptake by the cells. Sea-dwelling organisms are suspended in a medium of remarkably constant composition, hence, studies conducted with marine organisms were carried out in filter sterilized seawater. Experiments done with A. aerogenes were carried out at 37°C although satisfactory labelling, at a slower rate, occurred at lower temperatures. Since many marine bacteria cannot grow or even survive at temperatures above 20°C (Carlucci and Pramer, 1959), it was of interest to know if appreciable labelling of marine bacterial cells would occur at lower temperatures. Using the marine pseudomonad B-16 as a test organism, it was found that the cells became labelled at either 20°C, 15°C or 4°C after 90 minutes incubation (Table X). The radioactivity retained by cell suspensions rose to a maximum then rapidly decreased with increasing cell concentration. This effect was noted at each temperature of incubation. Succeeding experiments indicated that a peak uptake of tracer occurred with a population density of approximately 500 cells of marine Pseudomonas B-16 per ml. (Fig. 7). A similar effect of increasing cell concentration upon the amount of radioactivity retained by cell suspensions had been demonstrated with suspensions of A. aerogenes (Fig. 4).
The effect of temperature and the number of organisms in suspension upon the uptake of $^{32}P$ by cells of marine Pseudomonas B-16 after an incubation period of 90 minutes.

<table>
<thead>
<tr>
<th>Temperature of incubation</th>
<th>Cells per ml.</th>
<th>Counts per minute</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>36</td>
<td>8928 ± 252</td>
</tr>
<tr>
<td>40</td>
<td>347</td>
<td>9900 ± 181</td>
</tr>
<tr>
<td>40</td>
<td>3520</td>
<td>7246 ± 293</td>
</tr>
<tr>
<td>150</td>
<td>36</td>
<td>8695 ± 261</td>
</tr>
<tr>
<td>150</td>
<td>347</td>
<td>10309 ± 229</td>
</tr>
<tr>
<td>150</td>
<td>3520</td>
<td>7518 ± 78</td>
</tr>
<tr>
<td>200</td>
<td>36</td>
<td>7751 ± 244</td>
</tr>
<tr>
<td>200</td>
<td>347</td>
<td>8130 ± 92</td>
</tr>
<tr>
<td>200</td>
<td>3520</td>
<td>5586 ± 326</td>
</tr>
<tr>
<td>Blank</td>
<td>0</td>
<td>5917 ± 236</td>
</tr>
</tbody>
</table>

Counts per minute readings are averages of triplicate samples, the radioactivity being detected with a Nuclear Chicago Gas Flow Detector.

Cells per milliliter were determined by the standard drop plate method.
FIGURE 7

A standard curve relating the uptake of $^{32}$P to the number of cells of marine Pseudomonas B-16 in suspension. Cell suspensions were incubated at 4°C for 150 minutes. Numbers of viable cells in suspension were determined by the standard drop plate technique.

1) Counts per minute are reported as averages of quintuplicate samples.
FIGURE 7

COUNTS PER MINUTE

x 10^2

0 10^1 10^2 10^3 10^4 10^5

CELLS PER MILLILITER
An experiment was designed to indicate the effect of the time of incubation upon tracer uptake by cell suspensions of the marine pseudomonad B-16 at 4°C and 15°C. A greater peak uptake of tracer was noted with cells of marine *Pseudomonas* B-16, incubated at 4°C, than with those incubated at 15°C, however, a longer incubation period was required for maximum labelling of the cells at the lower temperature (Fig. 8). Tracer uptake by the cell suspensions increased to a maximum after a 90 minute incubation period at 15°C and a 150 minute incubation period at 4°C, then began to decrease. A similar effect of length of incubation period upon the amount of tracer retained by cells had been noted with *A. aerogenes* (Fig. 4).

**THE UPTAKE OF P-32 BY VARIOUS MARINE BACTERIA**

Time and temperature of incubation had been shown to affect similarly the extent of P°32 labelling of cells of the marine pseudomonad B-16 and a terrestrial organism, *A. aerogenes*. It was of interest to know if the marine pseudomonad B-16 was representative of the marine microbial population in its ability to take up P°32. To examine this question, several marine bacteria were investigated. The marine bacterial species chosen for this study included two marine pseudomonads, B-16 and B-10, *Cytophaga* B-9, *Vibrio* MB-22, *Photobacterium fisheri* and a Gram-negative, filamentous species, *Leucothrix mucor*. 
The effect of time and temperature of incubation upon the uptake of \( P^{32} \) by a cell suspension of marine *Pseudomonas* B-16.

Cells suspended at a level of approximately 432 cells per ml.

Radioactivity on the filters detected by a Nuclear Chicago Gas Flow Detector.

1. Incubation temperature was 40C.
2. Incubation temperature was 15C.
3. Control, no cells, 15C.
**Figure 8**

![Graph showing counts per minute over time](image)

- **Counts per Minute**: The y-axis represents counts per minute, ranging from $6 \times 10^3$ to $13 \times 10^3$.
- **Time (hours)**: The x-axis represents time in hours, ranging from 0 to 3.

Line 1:
- Starts at $6 \times 10^3$ at time 0.
- Increases and peaks at approximately $13 \times 10^3$ at time 3.

Line 2:
- Starts at $6 \times 10^3$ at time 0.
- Increases and peaks at approximately $10 \times 10^3$ at time 2.

Line 3 (dashed):
- Starts at $6 \times 10^3$ at time 0.
- Remains nearly flat, indicating stability.

Legend:
- Line 1: Solid line
- Line 2: Solid line
- Line 3: Dashed line
Also studied was a Gram-positive coccus, Gaffkya homari. Serial dilutions of those organisms in sterile seawater were incubated in filter sterilized seawater with $^32$P added as inorganic orthophosphate for one and two hour periods at 15°C. Radioactivity retained by the cells was determined as previously described. It was found that the cells of $G$. homari and $P$. fisheri, after a one hour incubation period, took up more tracer than did the cells of B-9, B-10 or Vibriom MB-22. Pseudomonas B-16 lay between these two groups in its $^32$P uptake (Fig. 9). The peak uptake of tracer occurred after a one hour incubation period for all organisms tested, except Cytophaga B-9 and Pseudomonas B-10. All organisms with the exception of B-9 and B-10, showed with increasing time of incubation, an increase in tracer uptake and then a sharp decrease. These results closely parallel those found with the terrestrial organism, A. aerogenes (Fig. 4). The time required for peak uptake of tracer was not determined for Cytophaga B-9 and the marine pseudomonad B-10. At the end of a one hour incubation period, the organisms tested varied in the $^32$P uptake over a four-fold range. The degree of difference in $^32$P content between the organisms after two hours of incubation had diminished such that they now varied over less than a two-fold range. The $^32$P uptake per unit cell of L. mucor was impossible to determine due to the filamentous character of this organism. Experiments were therefore undertaken to
FIGURE 9

The rate of uptake of $P^{32}$ by suspensions of cells of marine bacteria incubated at 15°C.

1. Gaffkya homari 70 cells per ml.
2. Photobacterium fisheri 62 cells per ml.
3. Vibrio MB-22 52 cells per ml.
4. Pseudomonas B-16 56 cells per ml.
5. Cytophaga B-9 71 cells per ml.
6. Pseudomonas B-10 70 cells per ml.

 Counts per minute reported as averages of quintuplicate samples.
determine if the uptake of \(^{32}\)P by this organism, on a dry weight basis, was similar to that of other marine bacteria.

THE RELATIONSHIP OF \(^{32}\)P UPTAKE TO THE DRY WEIGHT OF CELL MATERIAL

*Leucothrix mucor* and the marine pseudomonad B-16 were each grown in Trypticase Seawater Broth for 18 hours at 25°C on the rotary shaker. Cells were harvested and washed as previously described and were resuspended in a final volume of 100 ml of filter sterilized seawater. The dry weights of these cell suspensions were determined by a method which took into consideration the interference of the weights of salts in the suspending fluid (Buckmire, personal communication).

It was found that the cells of *L. mucor* took up more tracer per mg. dry weight of cell material than did the cells of *Pseudomonas* B-16 (Table XI). The amount of tracer retained by the cells rapidly decreased as the amount of cell material in suspension increased. Cells of *L. mucor* took up approximately four times the amount of \(^{32}\)P per mg. dry weight of cell material as was taken up by cells of marine *Pseudomonas* B-16. Since previous work (Fig. 7) has shown that when marine pseudomonad B-16 is present in suspension at a level of 111 cells per ml., the cell concentration is in the range for maximum \(^{32}\)P uptake per cell and since *L. mucor* was tested at an even lower concentration, as is indicated by the relative dry weight of cell material present, the comparison of the \(^{32}\)P uptake per unit
The relation of the uptake of $^{32}\text{P}$ by the cells of marine

Pseudomonas B-16 and of *Leucotarix mucor* to the dry weight

of cell material present.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Dry weight of cell material (mg/ml)</th>
<th>Cells/ml</th>
<th>cpm</th>
<th>cpm/mg cell material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas B-16</td>
<td>98x10^{-4}</td>
<td>11,400</td>
<td>3233 ± 90</td>
<td>3.3 x 10^5</td>
</tr>
<tr>
<td>&quot;</td>
<td>98x10^{-5}</td>
<td>1247</td>
<td>5052 ±100</td>
<td>5.1 x 10^6</td>
</tr>
<tr>
<td>&quot;</td>
<td>98x10^{-6}</td>
<td>111</td>
<td>5659 ± 72</td>
<td>5.7 x 10^7</td>
</tr>
<tr>
<td>L. mucor</td>
<td>28x10^{-4}</td>
<td>-</td>
<td>2345 ±110</td>
<td>8.3 x 10^5</td>
</tr>
<tr>
<td></td>
<td>28x10^{-5}</td>
<td>-</td>
<td>2375 ±165</td>
<td>8.3 x 10^6</td>
</tr>
<tr>
<td></td>
<td>28x10^{-6}</td>
<td>-</td>
<td>2607 ±117</td>
<td>22.8 x 10^7</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>0</td>
<td>2693 ± 64</td>
<td>-</td>
</tr>
</tbody>
</table>

Counts per minute reported as averages of quintuplicate samples.

Cells per ml determined by the standard drop plate method.
weight of cell material for the two organisms is probably valid.

THE EFFECTS OF PREFILTRATION UPON A CELL POPULATION

Since raw seawater from the euphotic zone contains varying numbers of large celled organisms, derived from the phyto and zooplankton (Wood, 1963), which could be expected to take up \(^{32}\text{P}\), the possibility of prefiltering seawater samples through a membrane filter which could be expected to retain the eucaryotic cells but which would permit the passage of bacterial cells, was considered.

Since it was considered probable that an 8 micron pore size filter would retain most of the large celled organisms, its capacity to affect the numbers of bacterial cells in suspension was investigated using suspensions of a pure culture of marine pseudomonad B-16. Cells of B-16 were suspended in filter sterilized seawater. Part of the suspension was prefiltered through an 8 micron pore size filter. The cells in suspension and those in the filtrate were incubated with \(^{32}\text{P}\) for 150 minutes at 4°C and then were sampled as previously described. These cell suspensions were also tested before and after filtration, by the drop plate method and the direct count technique. The results shown in Table XII indicate that prefiltration removed a significant proportion of bacterial cells from suspension. Drop plate counts and direct counts were less after filtration of the cell suspensions. The \(^{32}\text{P}\) tracer technique gave indications of a
TABLE XII

Effects of prefitering upon the cell population of a marine Pseudomonas B-16 cell suspension.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cells per ml.</th>
<th></th>
<th>Radioactivity (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Drop plate count</td>
<td>Direct count</td>
<td></td>
</tr>
<tr>
<td>Before filtering</td>
<td>47</td>
<td>52</td>
<td>10,638 ± 175</td>
</tr>
<tr>
<td></td>
<td>432</td>
<td>401</td>
<td>12,500 ± 207</td>
</tr>
<tr>
<td></td>
<td>4440</td>
<td>4700</td>
<td>8928 ± 111</td>
</tr>
<tr>
<td>After filtering</td>
<td>35</td>
<td>39</td>
<td>7421 ± 89</td>
</tr>
<tr>
<td></td>
<td>277</td>
<td>312</td>
<td>9573 ± 101</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>2875</td>
<td>10,111 ± 131</td>
</tr>
<tr>
<td>Blank</td>
<td>0</td>
<td>0</td>
<td>5917 ± 64</td>
</tr>
</tbody>
</table>

Prefiltration procedure consists of passing the cell suspension through an 8 micron pore size filter.

The radioactivity readings are averages of quintuplicate results.
decrease in cell population density also. Prior to filtration, increasing dilution of the cell suspension brought about an increase in $P^{32}$ retained by the sample dilution. Considering the 4000 cells per ml. level, filtration of the suspension brought about a further increase in $P^{32}$ retention by the cells. Such an effect would indicate a decrease in cell concentration (Fig. 7). It was calculated that approximately 30% of the B-16 cells in suspension, as monitored by the direct count method, were removed by the prefiltration procedure.

DETERMINATION OF A VIABLE BACTERIAL CELL COUNT IN SEAWATER USING THE P-32 TECHNIQUE

Preliminary experiments with marine Pseudomonas B-16 had established that the cells became labelled at either 15°C or 4°C but that a greater period of time was required for maximum labelling of the cells at 4°C (Fig. 8). When aliquots of a sample of raw seawater were incubated with $P^{32}$ at 4°C and 15°C and aliquots of these incubation mixtures were filtered at various times, the amount of radioactivity retained by the filters was found to vary with time and temperature in the same way as when a suspension of marine Pseudomonas B-16 was incubated with $P^{32}$ at the same time (Fig. 10). The raw seawater was not prefiltered. A direct microscopic count indicated the presence of 386 bacterial cells per ml. of raw seawater diluted 1:10 with filter sterilized seawater and revealed only a few plankton bodies. The drop plate method gave a count of
The effect of time and temperature of incubation upon the uptake of $^{32}$P by a suspension of cells of marine *Pseudomonas* B-16 and raw seawater.

1. *Pseudomonas* B-16 cells suspended at a level of approximately 432 cells per ml.

2. Raw seawater diluted 1:10 with filter sterilized seawater.

3. Control, no cells.

Radioactivity retained by the filters was detected with a Nuclear Chicago Gas Flow Detector.
20 cells per ml. of the 1:10 dilution. When the seawater sample was heated before incubation, retention of $^{32}$P by the filters rose only slightly above that retained by filtering the $^{32}$P containing incubation medium alone. The results suggested that the mixed microbial population in the seawater sample was responding to $^{32}$P in a manner similar to the cells of marine Pseudomonas B-16. If one assumes from the amount of radioactivity retained by the filters that the seawater dilution used, contains the same number of cells as the suspension of the marine pseudomonad used for comparison, one can calculate that the original undiluted seawater contained 4300 cells per ml. This compares with a direct count value of 3860 cells per ml, and a plate count of 200.

In an effort to enumerate bacteria in seawater using the $^{32}$P technique, it was decided to use marine Pseudomonas B-16 as the standard organism. A seawater sample was diluted as required using sterile seawater. At the same time, a washed suspension of marine pseudomonad B-16 was diluted with filter sterilized seawater from the same source. Both the seawater sample dilutions and the suspensions of marine pseudomonad B-16 were incubated with $^{32}$P. Aliquots of the suspensions were heated and incubated with $^{32}$P to determine the extent of non-specific binding of $^{32}$P. At the same time, drop plate and direct microscopic counts were made on all suspensions. From the number of marine pseudomonad
cells in suspension and the $^{32}\text{P}$ uptake on the filter, a standard curve was constructed (Fig. 7 and Fig. 11). The incubation mixtures were sampled as previously described and the results, expressed as counts per minute per ml. of sample filtered, were applied to the standard curve.

Previous experiments had shown that a low level of $^{32}\text{P}$ retained by a filter could be an indication of either a very large or a negligible cell density. The magnitude of the microbial population in the seawater samples was not known. Two methods were investigated in order to bracket the cell density of a particular sample. A 1/10 or a 1/100 or other dilution of a sample, as required, was incubated with $^{32}\text{P}$. In some cases, a known number of cells of Pseudomonas B-16 were added to an incubation mixture and their effect upon the radioactivity retained by the sample was noted. Results were interpreted as follows. If upon increasing dilution of a sample, the counts per minute per ml. of sample filtered decreased, the radioactivity readings could be said to be on the left hand side or ascending portion of the standard curve. If upon increasing the dilution of a sample, the radioactivity readings increased, the reading could be said to be on the right hand side or descending portion of the standard curve. Readings placed on the descending portion of the standard curve were not considered valid except in the upper portion of the curve where cell count estimates could be made within reasonable limits. Similar reasoning was used during the interpretation of the effect of
A standard curve relating the uptake of $^{32}$P to the number of cells of marine *Pseudomonas* B-16 in suspension. Cell suspensions were incubated at 4C for 150 minutes. Radioactivity was detected with a Nuclear Chicago Gas Flow Detector.

1) Numbers of viable cells in suspension were determined by the standard drop plate count method.
additions of known numbers of cells of marine Pseudomonas B-16 to an incubation mixture. If upon addition of 50 cells per ml. to an incubation mixture, the radioactivity retained by the filters increased, the reading was placed on the ascending portion of the standard curve. Likewise, if upon addition of the cells, the radioactivity retained by the filters decreased, the reading was placed on the descending portion of the standard curve. Two or more points were required before the placement of the radioactivity reading of a sample on the curve could be considered valid.

Results in Table XIII were obtained from two samples taken from the open ocean off Terence Bay, Nova Scotia and tested within two hours of collection. All radioactivity readings obtained with these samples were referred to the standard curve shown in Figure 11 which was prepared at the same time.

SAMPLE 1 - A 1/10 dilution of this sample gave 14,084 counts per minute. Upon further dilution of the sample, the radioactivity reading decreased, therefore, the 1/10 dilution reading and the radioactivity reading for the 1/100 dilution were placed on the ascending portion of the standard curve. Since there could be some question as to the placement of the 1/10 dilution reading, i.e. that the reading could be at the peak of the standard curve, the 1/100 dilution results only, were considered valid. The viable cell estimate obtained by the P technique was greater than the count obtained by the drop plate technique yet was lower than the cell count obtained by direct microscopic examination.
TABLE XIII

Bacterial cell counts of seawater samples, tested within two hours of collection, as determined by the Standard Drop Plate method, Direct Microscopic examination and the $^{32}$P tracer technique.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution</th>
<th>Radioactivity (cpm)</th>
<th>$^{32}$P mtd.</th>
<th>Direct ct.</th>
<th>Drop plate ct.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1/10</td>
<td>14,084</td>
<td>-</td>
<td>-</td>
<td>3800</td>
</tr>
<tr>
<td></td>
<td>1/100</td>
<td>9708</td>
<td>7600</td>
<td>29,376</td>
<td>3990</td>
</tr>
<tr>
<td>2</td>
<td>1/10</td>
<td>12,820</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1/100</td>
<td>8474</td>
<td>5200</td>
<td>27,400</td>
<td>4600</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>6060</td>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The radioactivity readings were applied to the standard curve shown in Figure 11. Radioactivity retained by the cells was detected with a Nuclear Chicago Gas Flow Detector.
SAMPLE 2 - Results obtained with this sample were similar to those obtained with Sample 1. Upon further dilution of a 1/10 dilution of a sample, the radioactivity decreased. This placed radioactivity readings on the ascending portion of the standard curve. Since only the 1/100 dilution was clearly on the ascending portion of the curve, it was chosen for comparison of the cell counts by the three different techniques. As with Sample 1, the drop plate count gave the greatest estimate of cell numbers. The P32 technique indicated a viable cell count between these two estimates.

Direct microscopic counts were readily obtained with both samples. Some bacterial cells could be seen to be adhering to strands of particulate matter (Plate I).

The second group of samples was collected on the morning of July 26, 1966, from the sea near Halifax, Nova Scotia, packed in a container with frozen, synthetic ice packs and shipped via Air Express to Montreal, arriving the same afternoon. The samples were immediately refrigerated and all further procedures were completed within 12 hours from the initial collection in the sea. The radioactivity measurements on the seawater samples were all referred to the standard curve shown in Figure 7, prepared at the same time. The results in Table XIV represent the data obtained with these samples.
PLATE I

Photomicrograph of a membrane filter after filtration of a seawater sample followed by staining of the particulate matter with Loeffler's Methylene Blue 1:5.

Magnification x 3000.
TABLE XIV

Bacterial cell counts of seawater samples air expressed from Halifax to Montreal, as determined by the Standard Drop Plate method, Direct Microscopic examination and the $^{32}\text{P}$ tracer technique.

<table>
<thead>
<tr>
<th>Sample Dilution</th>
<th>Treatment</th>
<th>Radioactivity (cpm)</th>
<th>$^{32}\text{P}$ mtd. Direct ct.</th>
<th>Plate ct.</th>
<th>% recovery of added B-16 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1/10</td>
<td>2484</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1/100</td>
<td>4880</td>
<td>8400</td>
<td>18,300</td>
<td>7260</td>
</tr>
<tr>
<td></td>
<td>1/100 plus cells</td>
<td>6132</td>
<td>19,000</td>
<td>24,100</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>123</td>
</tr>
<tr>
<td>2</td>
<td>1/5</td>
<td>3532</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1/10</td>
<td>2627</td>
<td>45</td>
<td>53,800</td>
<td>2346</td>
</tr>
<tr>
<td></td>
<td>1/100</td>
<td>1932</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1/10 plus cells</td>
<td>4069</td>
<td>450</td>
<td>59,600</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>82</td>
</tr>
<tr>
<td>3</td>
<td>1/5</td>
<td>3603</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1/10</td>
<td>2424</td>
<td>30</td>
<td>-</td>
<td>5460</td>
</tr>
<tr>
<td></td>
<td>1/100</td>
<td>2215</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1/10 plus cells</td>
<td>4140</td>
<td>460</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>86</td>
</tr>
<tr>
<td>4</td>
<td>1/5</td>
<td>2233</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1/10</td>
<td>4761</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1/100</td>
<td>6279</td>
<td>12,000</td>
<td>10,400</td>
<td>8000</td>
</tr>
<tr>
<td></td>
<td>1/100 plus cells</td>
<td>7590</td>
<td>-</td>
<td>15,500</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>1/10</td>
<td>5004</td>
<td>900</td>
<td>15,500</td>
<td>2550</td>
</tr>
<tr>
<td></td>
<td>1/100</td>
<td>2451</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1/10 plus cells</td>
<td>6527</td>
<td>2250</td>
<td>16,110</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>62</td>
</tr>
<tr>
<td>Blank</td>
<td></td>
<td>2455</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

The radioactivity readings were applied to the standard curve shown in Figure 7. Wherever "plus cells" is noted, 50 cells of marine Pseudomonas B-16 have been added to the sample dilution. The comparable number of added cells to undiluted sample is 500 cells per ml. to a 1/10 sample dilution and 5000 cells per ml. to a 1/100 sample dilution.
SAMPLE 1 - A 1/10 dilution of this sample gave a radioactivity reading of 2484 cpm. This reading increased to 4880 cpm upon increasing dilution of the sample to 1/100. Such an increase would place the latter reading on the ascending portion of the standard curve. This placement was confirmed by noting the effect of the addition of 50 cells of *Pseudomonas* B-16 to the 1/100 sample dilution, which resulted in an increase in radioactivity retained by the filters. Considering the 1/100 sample dilution results as valid, the $^{32}$P technique estimated the viable cell population of the sample as being 8400 cells per ml. This value was greater than the drop plate count and less than the direct count. A 123 per cent recovery of the added marine pseudomonad *B*-16 cells was noted with the 1/100 sample dilution.

SAMPLE 2 - A 1/5 dilution of this sample gave 3532 cpm. This reading decreased to 2627 cpm upon further dilution of the sample to 1/10. A further decrease in radioactivity was noted with a 1/100 dilution. It was therefore concluded that the reading for the 1/10 dilution was on the ascending portion of the standard curve. Addition of 50 cells of marine *Pseudomonas* B-16 per ml. to the 1/10 sample dilution caused an increase in the radioactivity reading thus confirming the position of the reading on the ascending portion of the standard curve. The $^{32}$P technique indicated the presence of only 45 cells per ml. of seawater sample while the drop plate and direct counts gave indications of the presence of many more cells. An 82 per cent recovery of the *Pseudomonas* B-16 cells added
to the 1/10 sample dilution was noted. Much particulate matter was observed during a direct microscopic examination of the sample.

SAMPLE 3 - This sample was similar to Sample 2 in that much particulate matter was present. A decrease in radioactivity with increasing dilution of the sample and an increase in radioactivity with decreasing dilution of the sample confirmed the validity of the 1/10 sample dilution reading. The $^{32}$P method indicated the presence of only 30 cells per ml. while the drop plate method gave a count of 5460 cells per ml. The direct count was not considered valid due to the presence of much particulate matter. Addition of 50 cells per ml. of marine Pseudomonas B-16 to the 1/10 sample dilution caused an increase in the radioactivity retained by the filters. The radioactivity reading obtained with the 1/10 sample dilution plus cells, when applied to the standard curve, indicated an 86 per cent recovery of the B-16 cells in suspension.

SAMPLE 4 - Upon increasing dilution of this sample, $^{32}$P retained by the filters increased, indicating that the 1/5 and 1/10 dilution radioactivity readings were on the descending portion of the standard curve. In order to ascertain the position of the 1/100 sample dilution reading, the effect of adding 50 cells per ml. of Pseudomonas B-16 to the 1/100 sample dilution was noted. The addition of cells brought about a further increase in radioactivity retained by the filters, however, this reading was slightly above the peak of the standard curve and therefore could not be applied to the
standard curve. It was concluded that the 1/100 sample dilution radioactivity reading was on the ascending portion of the standard curve. The $^{32}$P technique indicated the presence of 12,000 cells per ml., a value in excess of both the drop plate count and the direct microscopic count.

SAMPLE 5 - A 1/10 dilution of this sample gave 50,444 cpm. The radioactivity retained by the filters increased upon addition of 50 cells per ml. of Pseudomonas B-16 to the 1/10 sample dilution, thus confirming the position of the radioactivity reading on the ascending portion of the standard curve. This reading, when applied to the standard curve indicated the presence of 900 viable cells per ml. of raw seawater sample, while the drop plate count was 2550 cells per ml. The direct count was also greater than the $^{32}$P count. Of the 50 cells per ml. of Pseudomonas B-16 added to the 1/10 sample dilution, 62 per cent were recovered by the $^{32}$P technique.

Direct counts were obtained with difficulty in some of the seawater samples. Much particulate matter was viewed, particularly in Samples 2, 3 and 5 (Plate II).
PLATE II

Photomicrograph of a membrane filter after filtration of a seawater sample followed by staining of the particulate matter with Loeffler's Methylene Blue 1:5.

Magnification x 3000.
DISCUSSION

The technique for detecting and enumerating cells described in this thesis is based upon the universal requirement of living cells for phosphorus. Nutritional conditions, time and temperature of incubation and the concentration of the labelled phosphate have been shown to affect the uptake of the tracer by cell suspensions of A. aerogenes. A chemically defined medium containing potassium, magnesium and glucose has been found to promote optimum uptake of the tracer by the cells. The components, when tested alone or in combination, were found to be required in specific concentrations. Potassium, magnesium and glucose have been shown to be required for phosphate uptake and polyphosphate accumulation in various organisms, however, no workers have developed a chemically defined incubation medium composed of these compounds in order to attain optimum uptake of phosphate by bacterial cells (see Harold, 1966, for a review). Other compounds investigated as possibilities for the incubation medium were Tris (hydroxymethyl) amino methane buffer at 0.05M concentration and cysteine. These compounds were found to inhibit the uptake of $P^{32}$ by A. aerogenes. Tris buffer at 0.05M concentration, has been shown to uncouple oxidative phosphorylation (Good, 1962). Siegenthaler, Belsky and Goldstein (1967) found that Tris buffer at 0.1M concentration, completely inhibited the uptake of phosphate by an obligately marine fungus. Cysteine, has not to our knowledge, been shown to inhibit the uptake of $P^{32}$ by A. aerogenes.
Time and temperature of incubation had a marked effect upon the uptake of the tracer by the bacterial cells. It was found that the time required to label cells optimally, increased as the temperature of incubation decreased. Results of experiments conducted by various workers have indicated an effect of time upon the uptake of phosphate by bacterial cells (Myers and McCready, 1964; Harold, 1964), however, no attempt has been made to correlate the effect of time and temperature on the rate and degree of uptake of phosphate by *A. aerogenes*.

The effect of the concentration of labelled phosphate upon its uptake by the cells of *A. aerogenes* was investigated. It was found that the optimum concentration of the tracer was one microcurie per ml. and that amounts above and below this concentration gave rise to a decrease in uptake of the labelled phosphate. A decrease in the amount of labelled phosphate retained per cell was noted with increasing cell concentration. In an effort to explain these responses, unlabelled phosphate as \((\text{NH}_4)_2\text{HPO}_4\) was added to the incubation medium in amounts, 10 and 100 times the calculated amount of phosphate present in the one microcurie per ml. of \(^{32}\text{P}\) used in the incubation medium. It was found that even ten times the amount of \(^{32}\text{P}\) added as unlabelled phosphate, markedly inhibited the uptake of the tracer. Previous studies concerning the level of \(^{32}\text{P}\) required to optimally label cells have been mainly concerned with maintaining viability and even pathogenicity of cells, while obtaining a reasonable
uptake of the tracer (Robson, 1964; Myers and McCready, 1964). An excess of an unlabelled compound in the presence of the same labelled compound is known to competitively inhibit the uptake of the labelled compound by cells (Cowie, Bolton and Sands, 1951). It has also been noted that the nutrition of cells prior to the labelling procedure, has a marked effect upon the uptake of the tracer (Harold, 1966). It has therefore been concluded that the level of phosphate, labelled or not, in the incubation medium, cannot be varied to increase the labelling of the cells.

The composition of the washing fluid used to wash the labelled cells free of extracellular tracer, was found to affect significantly, the retention of the label by the cells. The chemically defined incubation medium, when used as a washing fluid in increasing volumes, was found to cause progressively increasing losses of $^{32}$P from the labelled cells. These results differ from those obtained with marine bacteria by Drapeau and MacLeod (1965), who found that the presence of specific cations in the washing fluid was required to prevent the release of intracellular solute from marine bacterial cells.

The method described in this thesis presents a number of problems and further studies will have to be done to evaluate the technique under all conditions. The amount of radioactive phosphate taken up by bacterial cells was found to vary with the concentration of cells in suspension. The lowest concentration of cells tested was 23 cells per ml, and this number of cells
could be detected with 95% confidence. Results in this study were obtained using pure cultures, grown under controlled conditions. The relative uptake of the tracer by individual members of a mixed population is not known. Studies have been conducted with resting cells of various organisms but the results of these experiments have not been compared to phosphate uptake in actively metabolizing cells. Some organisms take up phosphate only in the presence of an oxidizable substrate, such as was discovered by Mallin and Kaplan (1959) in their study of the resting cells of *Clostridium perfringens*. The assimilation of labelled phosphate by cells of *Serratia marcescens* was found to be dependent upon the nutritional state of the organism at the time of labelling (Myers and McCready, 1964). The nutritional state of the various organisms in a natural environment is extremely variable. Since the method described in this thesis utilizes a standard curve as an index of the number of viable cells present in a sample, it would seem necessary to use a standard curve prepared under the same conditions as unknown organisms in order to obtain a valid count. It may be that organisms, osmotically competent yet "dead" as far as their ability to reproduce was concerned, might still take up significant amounts of labelled phosphate.

Cell suspensions of *A. aerogenes* were treated with heat, formaldehyde and 2,4-dinitrophenol (DNP) in order to determine whether or not the treated cells would take up labelled phosphate.
Cells killed by heat or formalin did not take up a significant amount of tracer. Uptake of the tracer was severely restricted in cell suspensions treated with the inhibitor of oxidative phosphorylation (DNP). Similar results have been noted during studies conducted with DNP and cells of *A. aerogenes* (Smith, Wilkinson and Duguid, 1954). It is therefore suggested that dead cells and cells blocked in phosphate uptake, will not take up sufficient tracer to interfere with a viable cell count except, perhaps where there is a predominance of dead cells in a sample. Boiled blanks were used throughout this study, as a control for adsorption of tracer on cell material and particulate matter in suspension. Blank readings were not subtracted from the readings obtained with the corresponding sample, because the uptake of phosphate by viable cells was found to vary with the number of cells in suspension.

The labelling of marine bacteria with $^{32}$P was found to be controlled by factors similar to those concerned with the optimal uptake of phosphate by *A. aerogenes*. Studies with the marine pseudomonad B-16 revealed that the time and temperature of incubation of the cells with the tracer affected the uptake of the labelled phosphate. An incubation period of 150 minutes at 4°C was found to be necessary for optimal labelling of cell suspensions of the marine pseudomonad B-16. It was of interest to note that the labelling of a suspension of cells of marine *Pseudomonas* B-16 varied with time and temperature in the same
way as an aliquot of a dilution of raw seawater, incubated with $^{32}$P. A wide variation in the initial rate of uptake of $^{32}$P was noted in a survey of several marine bacterial species. There was as much as a four-fold difference in the amount of $^{32}$P taken up by the various species after a one hour period of incubation. Less than a two-fold difference in $^{32}$P uptake was demonstrated after a two hour incubation period. The marine pseudomonad B-16 was intermediate in its ability to take up $^{32}$P. For these reasons, the marine pseudomonad B-16 was chosen as a standard marine organism for further studies of seawater.

All studies concerning the uptake of $^{32}$P by marine organisms were carried out in filter sterilized seawater as an incubation medium. This medium was chosen because of the remarkably constant composition of seawater. Filter sterilized seawater was also used as a washing fluid when freeing labelled cells of extracellular $^{32}$P. It had been shown by Drapeau and MacLeod (1965) that the salts mixture optimum for uptake of intracellular solutes was also optimum for the prevention of the release of such compounds from cells of a marine pseudomonad.

The $^{32}$P technique was used to estimate the viable cell count in two groups of seawater samples, taken from the Atlantic Ocean off Terence Bay, Nova Scotia. The first two samples were collected from the sea, packed in a chilled, ice packed styrofoam insulation chest and were returned to the laboratory where they were tested. All experiments with these samples
were completed within two hours of collection. The temperature of the samples during transportation from the sea was noted to be 4°C. The second group of samples was collected on the morning of July 26, 1966, packed in a cardboard box with frozen synthetic ice packs and was shipped Air Express from Halifax, arriving in Montreal that afternoon. Unusually hot weather caused the ice packs to thaw during transit. As a result, the samples were exposed to temperatures greater than 4°C. Growth and multiplication of some organisms and the death of cells may have occurred. Clumping of organisms and much particulate matter was noted during direct microscopic examination of the second group of samples (Plate II).

The $^{32}$P tracer technique establishes an entirely different concept of a microbial population. The technique is merely presenting a measure of a given population by virtue of an individual cell's ability to take up radioactive phosphate. Knowing that only viable cells take up significant amounts of tracer, it may be said that the method is presenting an estimate of a viable population as a function of the chosen standard organism. A survey of seven marine bacterial species as to their phosphate uptake showed the standard organism, marine *Pseudomonas* B-16 as being intermediate in its rate of uptake. The uptake of $^{32}$P by the marine pseudomonad in response to time and temperature was similar to the uptake of the tracer by raw seawater. Numbers of cells of the standard organism were also determined by a standard drop plate.
technique which evaluated viable cells and a direct microscopic count which determined the total number of cells. Cells of Pseudomonas B-16 were readily counted by these methods. Both methods have inherent errors which are especially evident when the methods are applied to a natural environment. Direct counts are liable to many sources of error. Particulate matter and clumping of organisms lead to highly questionable results (Jannasch and Jones, 1958). Such clumping of organisms was noted particularly with the samples received by Air Express. Some direct counts of these samples were possibly underestimates of the total bacterial population for suspicious particles and clumps were not counted or estimated. Drop plate counts and plating procedures often give underestimates of the viable population of seawater (Kriss, 1963). This study bore up the premise in that all the seawater samples examined, gave a lower cell count on plates than was shown by direct count methods. Cell count estimates obtained upon application of the P$^{32}$ counting technique to the samples gave varied results. Both samples tested in Group 1, those obtained and examined in Halifax, gave P$^{32}$ cell counts which placed them between the drop plate count and the direct count. These seawater samples were not prefilted through 8 micron pore size membrane filters. A direct count of the samples revealed the presence of small groups of organisms adhering to strands of particulate matter while other organisms occurred singly. It was thought that
prefiltration procedures might discriminate against these organisms by removing them from the samples. It was considered a possibility that the organisms attached to a substrate might represent a unique part of the marine microflora. Since few plankton bodies were seen in these samples, it was decided to use the raw seawater, unfiltered, for experimental purposes. Bacterial cell counts by all methods compared favourably with those counts of bacteria in offshore waters that have been obtained by various workers (Kriss, 1963; Jannasch and Jones, 1959). Much particulate matter and clumping of organisms was noted upon examination of the second group of seawater samples which had been air expressed from Halifax to Montreal. Of the five samples tested in this group, three had viable cell counts by the P³² technique which were smaller than the cell counts obtained with the drop plate technique. Each of these samples was noted to have much particulate matter and clumping of cells (Samples 2, 3 and 5; Table XIV). It was of interest to note that the cells of marine Pseudomonas B-16, when added to dilutions of these samples, were readily and accurately detected. Percent recovery of the B-16 cells varied from 62 to 123% and averaged at 80% recovery of the added cells. This finding would tend to discount the possible presence in these seawater samples of inhibitors of the uptake of P³² by the organisms contained therein and would serve to indicate that there were many organisms
in the samples which did not take up $^{32}\text{P}$ at a rate comparable
to that of the standard organism. The samples in the second
group were not prefiltered to remove phytoplankton. Few
plankton cells were viewed during direct microscopic counts.
It has been noted by Wood (1963) that small celled plankton
are present in seawater in varying concentration. These cells,
called "nannoplankton" are of a size range of 1 micron and
would certainly pass through an 8 micron pore size filter. It
has been observed by Lasker and Holmes (1957), that cells of
Dunaliella sp., 6 to 9 microns in diameter, would pass through
an 8 micron pore size filter and could be retained by a 0.45
micron pore size filter. Photosynthetic algal cells take up
phosphate at a much reduced rate in the absence of light
(Kylin, 1966). The effect of photosynthetic eucaryotic cells in
a seawater sample can perhaps be reduced by incubating the
samples in the dark.

The samples of seawater tested during this study were too
few in number to give much more than an indication of the
possibilities of the $^{32}\text{P}$ method to enumerate viable bacteria
in seawater. Further development of the method will be required to
make it useful under any enviromental condition. Studies with
pure cultures of A. aerogenes and marine Pseudomonas B-16
have shown the method to be feasible. Similarities in the
manner of uptake of $^{32}\text{P}$ have been noted with all organisms
investigated. Time and temperature of incubation were shown to similarly affect the uptake of $P^{32}$ by suspensions of cells of marine *Pseudomonas B-16* and of raw seawater. All organisms surveyed showed upon increasing time of incubation, an increase in $P^{32}$ uptake followed by a decrease. The similarities of response to $P^{32}$ between the terrestrial *A. aerogenes*, marine *Pseudomonas B-16* and other marine organisms suggest the feasibility of using a standard organism as an index of viable cells. Organisms used in this study were grown under optimum conditions. Careful consideration will have to be given to the choice of the standard organism and the physiological state of cells at the time of preparation of the standard curve, if such a curve is to be considered valid.
SUMMARY

1. A new method has been discovered for the detection and enumeration of viable bacterial cells. It depends upon the ability of viable cells to become radioactive when incubated with $P^{32}$ labelled inorganic orthophosphate. When the radioactive cells were filtered from the suspending medium, the amount of radioactivity retained by the filter over a limited range of cell concentration could be related to the number of cells in suspension.

2. Optimal conditions for the uptake of $P^{32}$ by cell suspensions of Aerobacter aerogenes were determined. For maximum rate of uptake of $P^{32}$ by the cells, $K^+$, $Mg^{++}$ and glucose had to be present together at appropriate concentrations.

3. Phosphate uptake by A. aerogenes was found to reach a maximum level then rapidly decrease with increasing numbers of cells in suspension and with increasing time of incubation. The rate of uptake increased as the temperature increased from 20C to 37C.

4. As few as 23 cells per ml. could be detected by the method with 95 per cent confidence.

5. Each of seven marine bacteria became radioactive when incubated in sterile seawater containing $P^{32}$ added as inorganic orthophosphate.
6. The time of incubation of the cells of the marine organisms and the concentration of cells affected the uptake of $P^{32}$ in a manner similar to that observed with *A. aerogenes*.

7. Uptake of $P^{32}$ by an aliquot of raw seawater was found to vary with time and temperature in the same way as when a suspension of marine *Pseudomonas B-16* was incubated with the tracer.

8. Using marine *Pseudomonas B-16* as a standard, the number of viable marine bacteria in several seawater samples was estimated and compared with counts obtained by conventional methods.
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