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Sensitive Rapid Detection Method for Viable Bacterial Cells

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ABSTRACT

MACLEOD, ROBERT A. (Macdonald College of McGill University, Ottawa, Ontario, Canada), MARILYN LIGHT, LLOYD A. WHITE, AND J. F. CURRIE. Sensitive rapid detection method for viable bacterial cells. Appl. Microbiol. **14**:979–984. 1966.—A rapid sensitive method for the detection of viable bacterial cells is described in which P^{32} as inorganic orthophosphate is used to label the cells. Factors affecting the uptake of P^{32} by cells as well as the sensitivity of the method have been explored with suspensions of *Aerobacter aerogenes*. The uptake of $P^{32}O_4$ is dependent on several factors. Of various incubation media tested, one composed of 0.005 M KCl, 0.002 M $MgSO_4$ and 10 mg/ml of glucose was found to best stimulate the uptake of the tracer. Incubation time and temperature and level of isotope and of unlabeled P also affected uptake. Labeled cells were collected on a membrane filter for measurement of radioactivity. Under optimal conditions, as few as 23 viable cells per milliliter were detected in 1 hr with 95% confidence.

It has been stated that many more bacteria are present in the sea, the soil, and other natural habitats than are capable of growing on laboratory media [*see* MacLeod (13) for a review]. Waksman et al. (20) observed that only about 0.1% of the bacteria in seawater or marine mud observed by direct microscopic examination are able to grow on plating media. Kriss (10) reported that standard media permitted the detection of not more than 0.1 to 1% of the total number of organisms which can be observed microscopically in seawater or mud samples. Similarly, it has been concluded that only a small percentage of the bacteria observed by microscopy in soil and freshwater ever grow on laboratory media [*see* Gibson (4) for a review].

The conclusion that viable counts detect only a part of the natural population is based largely on comparisons with direct microscopic counts. The latter, as ordinarily applied, do not distinguish between living and dead cells. Efforts to make such a distinction by the application of vital staining techniques [*see* Strugger (19)] have been found, at least in certain circumstances, not to be successful (9). For this reason, alternative methods of measuring viable cells should be explored to determine whether better measures of the number of cells in natural habitats can be obtained.

Methods for the detection of viable cells by use of radioactive isotopes have been reported. Levin (12) devised a method which depends on the capacity of cells to metabolize a radioactive substrate, giving rise to a product which separates as a radioactive gas. This procedure suffers from the disadvantage that no single substrate is universally metabolizable, and hence the procedure could not be adapted for use in obtaining total counts of mixed populations of bacteria. The extent of $C^{14}O_2$ assimilation by photosynthetic microorganisms has been used as a measure of primary productivity in the sea (15). Such a method has obvious limitations for use with nonphotosynthetic bacteria.

Many substances can be concentrated inside living cells by means of energy-dependent transport processes to levels which in some cases are many times higher than those prevailing in the suspending medium (8). If the substances are radioactive, then the cells accumulate radioactivity. The cells can be separated from the suspending medium by use of a membrane filter, and their radioactivity can be detected by standard methods (1, 5). It seemed worthwhile to explore the possibility of using the ability of cells to concentrate isotopes to develop a sensitive method of detecting viable cells. If the method is to be used to obtain a total count, a substrate

required by all types of bacterial cells would be needed. For this reason, we have examined the possibility of using P^{32}, added in the form of inorganic phosphate, as a labeling agent.

As a preliminary to more extensive investigations, it was considered necessary to determine the feasibility of using P^{32} labeling to detect viable bacterial cells. For this purpose, a pure culture of *Aerobacter aerogenes* has been used, and some of the factors affecting the accumulation of the radioactive isotope by the cells have been examined. It has been found that cells of this organism can be rapidly labeled with P^{32} and readily detected in very low concentration.

MATERIALS AND METHODS

Organism. The organism used was *A. aerogenes*, Macdonald College Culture Collection No. 112. It was carried on slants of Trypticase Soy Agar (BBL) and was transferred monthly.

Growth and harvesting of cells. One loopful of cells from a slant was used to inoculate 200 ml of Trypticase Soy Broth (BBL) in a 2-liter conical flask. The cells were grown on a rotary shaker at 30 C for 24 hr. The cells were harvested by centrifugation at 16,300 × *g* and 4 C, and were washed three times by centrifugation from, and resuspension in, sterile glass-distilled water (GDW). The suspension of washed cells was adjusted to a level of approximately 10^8 cells per milliliter by referring the turbidity of a sample of the suspension to a previously calibrated curve relating numbers of cells to turbidity. Serial dilutions of the suspension were made with sterile GDW to give the desired cell concentrations for the experiments.

P^{32} source. Carrier-free radioactive phosphorus as $H_3P^{32}O_4$ (*p*H < 1) was obtained from Atomic Energy of Canada Ltd., Ottawa, Ont., Canada. The volume received was neutralized by dilution with 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.2). Since this radioactive isotope has a short half-life (14.3 days), a computation of the actual amount of radioactivity present in the source was made before each experiment. A sample of the P^{32} source was then diluted with sufficient 0.05 M Tris buffer to give 10 μc of P^{32} per ml. This solution was sterilized by filtration with a sterile membrane filter (type HA, Millipore Filter Corp., Bedford, Mass.).

Incubation medium and labeling procedure. The composition of the incubation medium is shown in Table 1. The KCl, $MgSO_4$, and glucose were dissolved in sufficient GDW to give a concentration one-fourth greater than the final concentration in the incubation medium. This solution was sterilized by autoclaving at 121 C for 15 min. To 8 ml of this solution, in a sterile 25-ml Erlenmeyer flask, were added 1 ml of P^{32} solution (10 μc/ml) and 1 ml of an appropriately diluted suspension of *A. aerogenes*. A blank without cells was prepared by substituting 1 ml of sterile GDW for the cell suspension. The flasks were incubated at 37 C in a rotary water-bath shaker for 1 hr, unless otherwise indicated.

Detection of P^{32} retained by the cells. A 1-ml portion of the incubation mixture was filtered through a sterile 25-mm HA Millipore filter held in a Pyrex microanalysis filter apparatus. [Acropor ANW filters (Gelman Instrument Co., Ann Arbor,, Mich.) have been found to be equally satisfactory.] The cells on the filter were then washed by passing 5 ml of sterile GDW through the filter. To avoid the possibility of contaminating the filter with airborne organisms, the top of the filter funnel was plugged with a rubber stopper. Air was drawn into the filter through a cotton-plugged glass tube fitted in the stopper.

TABLE 1. *Composition of incubation medium*

Component	Concn
KCl	0.005 M
$MgSO_4$	0.002 M
Glucose	10 mg/ml
P^{32} (in 0.05 M Tris buffer)	1 μc/ml

Each incubation mixture was sampled in quintuplicate. Portions (1 ml) of the incubation medium prepared without cells were treated in the same manner as those containing cells, to serve as blanks. Since only a limited number of filter holders were available, the funnel of the filter apparatus was immersed for 1 min in 70% ethyl alcohol and then rinsed in sterile GDW between samplings.

After the filtrations and washings, each filter was placed on a clean planchet and air-dried. The radioactivity retained on the filters, both in the case of the samples containing cells and the blanks, was determined with a thin end window Geiger-Müller tube attached to a Picker scaler. The scaler was preset to record 4,000 counts, and the time to accumulate this number of counts in the case of each sample was determined. Results, unless otherwise indicated, were recorded as counts per minute per milliliter 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 with Trypticase Soy Agar as the plating medium. To obtain a count valid to within a coefficient of variation of 7%, a minimum of 400 colonies were counted for each sample.

RESULTS

Effect of composition of the incubation medium on extent of labeling of the cells. Various compounds were added to suspensions of cells in distilled water to determine their effect on the rate and extent of labeling of the cells by P^{32}, added as inorganic phosphate. The choice of compounds for study was governed by a consideration of those compounds which might be expected to be most concerned with the stimulation of P^{32} uptake. Resting cells of *Clostridium perfringens* have been shown to require either ethyl alcohol or glucose for P^{32} incorporation (14). Solomon (17) suggested that K^+ is linked

TABLE 2. *Effect of the various components of the incubation medium, tested alone and in combination, on the uptake of P^{32} by cells of Aerobacter aerogenes*

Additions to GDW	Radioactivity on filter	
	No cells	Plus cells[a]
None	2,560 ± 51	3,759 ± 101
KCl (0.005 M)	2,890 ± 144	4,540 ± 118
$MgSO_4$ (0.002 M)	2,701 ± 81	3,281 ± 114
Glucose (10 mg/ml)	3,035 ± 136	3,819 ± 114
KCl + $MgSO_4$	2,790 ± 68	4,162 ± 124
KCl + glucose	2,666 ± 79	4,789 ± 143
$MgSO_4$ + glucose	2,956 ± 144	3,759 ± 75
KCl + $MgSO_4$ + glucose	2,857 ± 71	5,121 ± 102

[a] Suspensions contained an average of 500 cells per milliliter as determined by drop plate count.

with phosphate metabolism in *Escherichia coli*. Mg^{++} has been shown to be important for the maintenance of respiratory activity in *Azotobacter* (7). The effect of K^+, Mg^{++}, and glucose, alone and in combination, on the uptake of P^{32} by cells of *A. aerogenes* was, therefore, examined. It is evident from Table 2 that cells suspended in distilled water became labeled with P^{32} and could be detected. KCl tested alone stimulated P^{32} incorporation; glucose alone was essentially without effect, and $MgSO_4$ was inhibitory. The most effective incubation mixture tested, however, was one in which KCl, glucose, and $MgSO_4$ were present together. Tris buffer (*p*H 7.2), tested at a level of 0.05 M, as well as cysteine at 10^{-3} M, were both found to be inhibitory. Tris buffer has been shown to uncouple electron transport from phosphorylation (6).

The optimal levels of KCl, $MgSO_4$, and glucose in the incubation medium 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^{32} uptake by *A. aerogenes*. A sharp maximum was observed at 0.005 M KCl. With this level of K^+ in the medium and 10 mg/ml of glucose, $MgSO_4$ was found to be most effective at 0.002 M (Fig. 2). The optimal concentration of glucose was found to be 10 mg/ml (Fig. 3).

Time required for labeling cells. With the optimal levels of incubation medium components as determined above, the time required for maximal uptake of P^{32} was found to be 60 min (Fig. 4). This experiment was conducted with approximately 500 cells per milliliter. We also have evidence to indicate that, when the number of cells is increased to 6,500 per milliliter, the time for

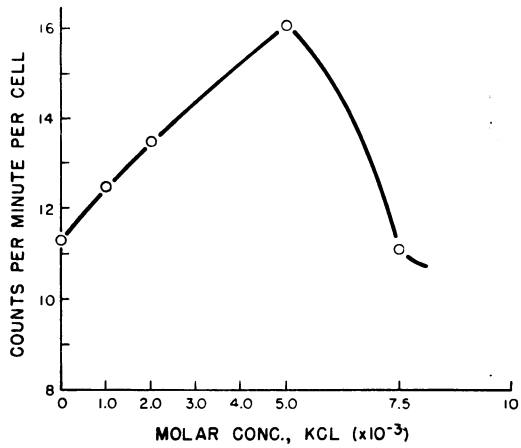

FIG. 1. *Effect of KCl concentration on the uptake of P^{32} by cells of Aerobacter aerogenes in suspension. Incubation medium contained 0.002 M $MgSO_4$ and 10 mg/ml of glucose. Cells suspended at a level of approximately 340 cells per milliliter.*

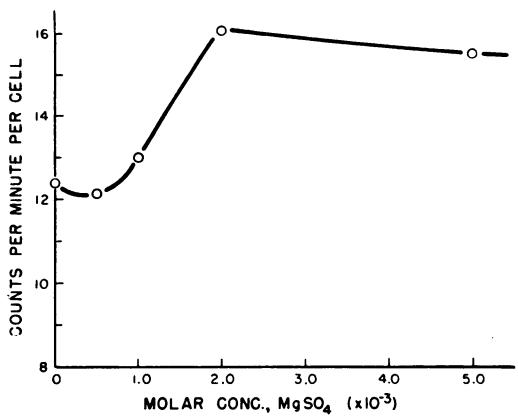

FIG. 2. *Effect of $MgSO_4$ concentration on the uptake of P^{32} by cells of Aerobacter aerogenes in suspension. Incubation medium contained 0.005 M KCl and 10 mg/ml of glucose. Cells suspended at a level of approximately 335 cells per milliliter.*

maximal labeling is increased to 90 min. Since most of our tests were conducted with 500 cells per milliliter, or less, the incubation period selected for further study was 1 hr.

Effect of temperature. Preliminary experiments had established that the most rapid labeling 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 labeling of the cells could occur at lower temperatures. The results in Table 3 show that cells of *A. aerogenes* became as extensively

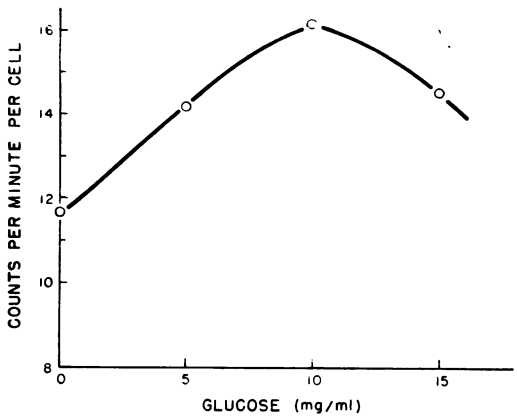

FIG. 3. *Effect of glucose concentration on the uptake of P^{32} by cells of Aerobacter aerogenes in suspension. Incubation medium contained 0.005 M KCl and 0.002 M $MgSO_4$. Cells suspended at a level of approximately 325 cells per milliliter.*

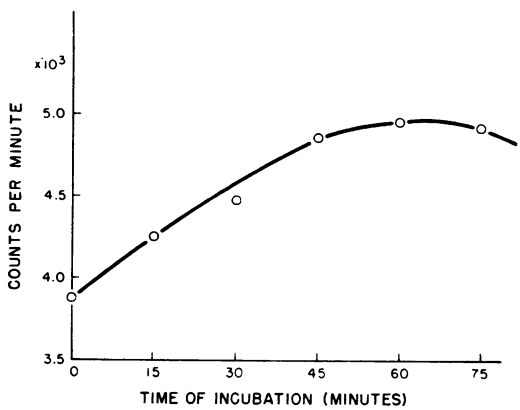

FIG. 4. *Effect of incubation time on the extent of labeling of cells of Aerobacter aerogenes in suspension. Cells suspended at a level of approximately 500 cells per milliliter.*

labeled at 20 C as at 37 C, but that, at the lower temperature, 100 rather than 60 min was required for the process.

Effect of P^{32} level. Various levels of P^{32} were tested in the incubation medium. The optimal uptake of P^{32} by the cells (Fig. 5) occurred with 1 μc/ml of incubation medium. Both above and below this level, the counts per min 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 solutes in the case of marine bacteria (3), the effect of washing on the retention of P^{32} was examined. With marine bacteria, conditions optimal for the uptake of substrates were also optimal for the

TABLE 3. *Effect of incubation temperature on the time required for maximal uptake of P^{32} by Aerobacter aerogenes*[a]

Incubation time	Incubation temp	
	20 C	37 C
min		
0	2,582 ± 175	2,593 ± 170
60	3,547 ± 571	5,012 ± 384
80	3,893 ± 541	4,914 ± 631
100	6,026 ± 533	—
115	4,673 ± 395	—
150	4,289 ± 145	—

[a] Suspensions contained an average of 900 cells per milliliter as determined by drop plate count.

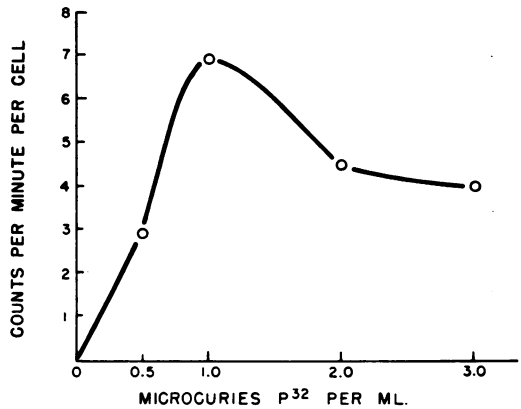

FIG. 5. *Effect of level of P^{32} in the incubation medium on the extent of labeling of cells of Aerobacter aerogenes in suspension. Cells suspended at a level of approximately 450 cells per milliliter.*

prevention of their release. Such was not the case with *A. aerogenes*, however (Table 4). Washing cells of this organism with increasing volumes of incubation medium caused progressively increasing losses of P^{32}. Washing with distilled water, however, did not remove the isotope. It is of interest to note that at least part of the decrease in radioactivity caused by washing with incubation medium is due to removal of P^{32} bound to the filter.

Limits of detectability of cells by means of P^{32} labeling. The range of cell numbers detectable by the P^{32} labeling technique was determined by observing the increase in P^{32} activity on the filters as the number of cells in suspension in the incubation medium was increased. The results (Fig. 6) show that the P^{32} count increased sharply as the number of cells increased from 0 to about 7,000 cells per milliliter, and then dropped off. The lowest concentration of cells tested was 23 cells

TABLE 4. *Effect of type and volume of washing fluid on the retention of P^{32} by labeled cells of Aerobacter aerogenes*[a]

Vol	Washing fluid			
	Glass-distilled water		Incubation medium[b]	
	Filter + cells[c]	Blank	Filter + cells[c]	Blank
ml				
5	5,012 ± 184	2,557 ± 154	6,031 ± 108	3,122 ± 98
10	4,199 ± 266	2,680 ± 258	3,459 ± 529	2,185 ± 454
15	4,910 ± 554	2,492 ± 91	1,567 ± 107	1,580 ± 163
20	5,137 ± 257	2,538 ± 43	1,803 ± 263	1,655 ± 116

[a] Results are expressed as counts per minute.
[b] The incubation medium used for washing was prepared without adding P^{32}.
[c] Suspensions contained an average of 800 cells per milliliter as determined by drop plate count.

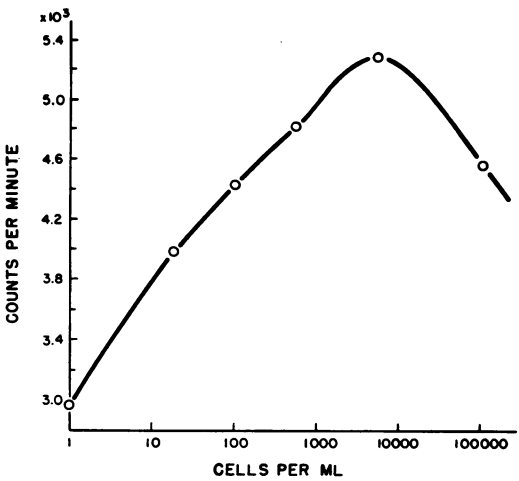

FIG. 6. *Effect of cell concentration on the radioactivity retained by the filters after incubating cell suspensions of Aerobacter aerogenes in the presence of P^{32}.*

TABLE 5. *Statistical analysis of the degree of significance to be attached to the P^{32} uptake technique for detecting cells when 23 cells per milliliter are present in suspension*[a]

Determination	X_1 (blank, no cells)	X_2 (23 cells/ml)
Values obtained	3,571	4,608
	2,861	4,866
	2,826	2,838
	2,921	4,819
	2,867	3,739
Mean	3,009	4,174
Standard deviation	315.9	872.0

[a] Student's t, calculated = 2.807. Students t, from tables: t .05, 8 = 2.306. Therefore, the means are significantly different at the 95% confidence level.

per milliliter, and the results in Table 5 show that this number of cells could be detected with 95% confidence.

Effect of unlabeled phosphorus. The results in Fig. 6 show that, though the P^{32} activity increased as the number of cells on the filter increased, the counts per cell decreased approximately logarithmically. Since there was a limit to the amount of P^{32} which could be added to the incubation medium (Fig. 5), the effect of adding unlabeled P was determined. It was calculated that the amount of phosphorus added as P^{32} was 3.49×10^{-8} g/ml. Unlabeled P as $(NH_4)_2HPO_4$ was

TABLE 6. *Effect of unlabeled phosphorus on P^{32} uptake by cells of Aerobacter aerogenes*

Cells/ml	Unlabeled P added (g/ml)[a]		
	0	3.49×10^{-7}	3.49×10^{-6}
0	2,783 ± 108	2,312 ± 69	2,774 ± 154
5.2 ± 1.1	3,565 ± 245	2,781 ± 162	2,182 ± 252
620 ± 13	4,629 ± 230	2,528 ± 200	2,643 ± 208
61830 ± 1.1	7,561 ± 225	3,392 ± 334	3,418 ± 170

[a] Unlabeled P added as $(NH_4)_2HPO_4$.

TABLE 7. *Effect of heating, formaldehyde treatment, and 2,4-dinitrophenol (DNP) on the capacity of cells in suspension to take up P^{32}*

Treatment[a]	Radioactivity on filter	Viable bacteria per ml
	count/min	
Blank	2,698 ± 304	—
Blank + DNP	2,334 ± 748	—
Blank + formaldehyde	2,638 ± 218	—
Cell suspension	5,144 ± 536	835
Cells + DNP	1,864 ± 405	789
Cells + formaldehyde	3,418 ± 294	0
Cells + heat	3,860 ± 406	0

[a] DNP concentration, 10^{-3} M; formaldehyde, 1%; heat treatment, 100 C for 5 min.

added to the incubation medium at 10 and 100 times this level. The results (Table 6) show that even 10 times the level markedly depressed the uptake of P^{32}, even though the amount of unlabeled P 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, formaldehyde, and 2,4-dinitrophenol (DNP) on the capacity of the cells to take up P^{32} was examined (Table 7). It is evident that the greatest amount of P^{32} uptake occurred with untreated cells, and the least was obtained in the presence of DNP. In both of these cases, the cells could be shown still to be viable on subsequent plating of the suspensions. Though no viable cells remained after heating or formaldehyde treatment, some uptake of P^{32} over the corresponding blank was obtained. It is also of interest to note that the radioactivity was less on filters through which the DNP-treated cell suspensions were filtered than in filters through which the incubation medium containing no added cells was filtered.

DISCUSSION

There have been many studies conducted on the labeling of bacteria with P^{32} during growth (11, 16, 18). Only a limited amount of information is available concerning the uptake of P^{32} by resting cells (14).

Spores which have been labeled with P^{32} during growth have subsequently been used as bacterial markers in various types of experiments (*see* 2). So far as we are aware, however, no previous attempt has been made to use P^{32} labeling as a technique to detect previously unlabeled cells. It is evident from the present study that, if carried out under appropriate conditions, the method described here is both rapid and sensitive, and could have wide application. It is also evident that certain factors can stimulate or interfere with P^{32} uptake. If the method is to be used for a total count on natural materials, appropriate internal standards will have to be employed. This aspect of the study is now being pursued.

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