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Method for Radiorespirometric Detection of Bacteria in Pure Culture and in Blood

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Methods are described for the detection of low numbers of bacteria by monitoring $^{14}CO_2$ evolved from ^{14}C-labeled substrates. Cell suspensions are filtered with membrane filters, and the filter is then moistened with 0.1 ml of labeled medium in a small, closed apparatus. Evolved $^{14}CO_2$ is collected with $Ba(OH)_2$-moistened filter pads and assayed with conventional radioactivity counting equipment. The kinetics of $^{14}CO_2$ evolution are shown for several species of bacteria. Fewer than 100 colony-forming units of most species tested were detected in 2 h or less. Bacteria were inoculated into blood and the mixture was treated to lyse the blood cells. The suspension was filtered and the filter was placed in a small volume of labeled medium. The evolved $^{14}CO_2$ was trapped and counted. A key development in the methodology was finding that an aqueous solution of Rhozyme and Triton X-100 produced lysis of blood but was not detrimental to bacteria.

The use of radioisotopes to detect the presence of microorganisms of medical significance was first reported by Levin et al. (7). The basic technique involved collection of bacteria on a membrane filter, immersion of the filter in a medium containing ^{14}C-labeled substrates, and collection of metabolically produced $^{14}CO_2$. Subsequent publications described the use of [*1-*^{14}C]lactose for a one-step, presumptive coliform test (8, 9) and the use of ^{14}C-formate in an inhibitory broth for a one-step, confirmatory fecal coliform test (10, 15). A quantitative relationship between evolved radioactivity and numbers of organisms was found.

Scott et al. (18, 19) confirmed the findings of the Levin group by using *m*-Endo broth containing ^{14}C-formate. Levin et al. have continued to develop the basic test as a means of life detection on other planets (5, 6, 11-14).

DeLand and Wagner (3) reported a radiometric method for the detection of bacterial growth in blood cultures. Their procedure involved the monitoring of $^{14}CO_2$ gas which was flushed from liquid culture bottles containing ^{14}C-labeled D-glucose. Washington and Yu (20) tested the method of DeLand and Wagner on simulated blood cultures and on a limited number of patient blood cultures. They reported that the radiometric method did not provide earlier evidence of bacteremia than did routine procedures and that it was impossible to detect 4 to 4,250 colony-forming units (CFU) within 6 h. Deblanc et al. (1) compared 2,967 blood cultures by conventional techniques and found that bacteria were detected more rapidly 70% of the time by the radiometric method. Waters (21) and Previte (16), by using an automated radiometric method, reported that detection times for various bacteria decreased with increased cell numbers. Depending upon species, inocula of 100 cells were detected in 6 to 14 h.

This publication describes modifications of the above-referenced coliform procedures for the detection of low numbers of various pathogenic bacteria in pure cultures and also in blood after lysis and filtration. Lysis of blood and filtration prior to radiorespirometric detection of bacteria is an important aspect of the procedure. Antibacterial agents present in blood are eliminated, evolution of $^{14}CO_2$ by blood cells which can mask detection of low numbers of bacteria is greatly decreased, low liquid volume and high specific activity of ^{14}C-labeled substrates promote a rapid response, and isolated colonies, which provide confirmation and material for isolation and sensitivity determinations, appear on the filter subsequent to positive detection.

Although the method has been developed specifically for detection of bacteremia, the

procedure is adaptable for spinal fluid and other filterable fluids for which a rapid sterility determination is needed.

MATERIALS AND METHODS

Cultures. Cultures of *Escherichia coli*, *Staphylococcus aureus*, hemolytic *Streptococcus*, *Salmonella paratyphi* B, *Haemophilus aphrophilus*, and *Cardiobacterium* sp. were supplied by James D. MacLowry of the National Institutes of Health. Cultures of *Pseudomonas aeruginosa*, *Klebsiella* sp., *Salmonella typhi*, *Shigella dysenteriae*, *Enterobacter aerogenes*, *Serratia marcescens*, and *Proteus vulgaris* were supplied by the Center for Disease Control, Atlanta, Ga. Cultures were maintained on Trypticase soy agar (TSA) with the exception of *H. aphrophilus* and *Cardiobacterium* which were maintained on TSA agar plus dextrose which had been enriched with 2 g of yeast extract, 20 mg of hemin, and 2 mg of nicotinamide adenine dinucleotide per liter. Eighteen to 24 h prior to an experiment, cultures were inoculated in broth medium and incubated at 37 C. Each culture was then serially diluted in Trypticase soy broth (TSB) medium before inoculation into the test system. Cell numbers were determined immediately before each experiment by spread plate techniques.

Lysis of blood. Normal blood specimens were supplied by the National Institutes of Health Clinical Center. They were drawn in 8-ml (yellow-cap) vacutainer tubes containing 0.05% sodium polyanathol sulfonate (Becton Dickinson).

Two techniques for lysing blood were principally used throughout the study. They were the modified technique of Rose and Bradley (17) and a Rhozyme procedure developed during this study. The former method was performed as follows. Blood (1 ml) was added to 19 ml of autoclaved, sterilized lysing solution (0.5 g of Triton X-100 [Sigma], 8 g of Na_2CO_3 per liter of water). The solution was allowed to stand for 3 or 4 min at room temperature and was then filtered through a 0.45-μm pore size membrane filter (Millipore Corp.). The filter was washed with 15 ml of 0.85% saline.

The Rhozyme method was performed as follows. Blood (3 ml) was added to 37.5 ml of lysing solution consisting of 4 ml of a stock solution (20 mg/ml, filtered and sterilized by membrane filtration) of Rhozyme 41 concentrate (Rohm and Haas), 1.5 ml of autoclaved 0.1% Triton X-100, and 32 ml of water. The mixture was incubated in a 37 C water bath for 30 min and then filtered through a 0.65-μm pore size 25-mm membrane filter. The filter was washed with 10 ml of sterile TSB. The concentrations of Triton X-100 and Rhozyme in the total volume of lysed blood were 0.04 mg/ml and 12.13 mg/ml, respectively.

Aliquots of the Rhozyme and Triton X-100 stock solutions were aseptically pipetted into screw-capped bottles of dilution water. These bottles of lysing solution were refrigerated and could be stored for at least 3 weeks without noticeable loss in lysing activity.

^{14}C-labeled medium. Radioactive medium was prepared in 10-ml batches as follows. To a sterile 25-ml vial were added: D-[*UL*-^{14}C]glucose, 60 μCi; [*1*-^{14}C]glu-conate, 20 μCi; [*UL*-^{14}C]glycine, 20 μCi; [^{14}C]formate, 20 μCi; and 0.1 ml of supplement B (Difco). Concentrated solutions of unlabeled substrates were added to bring the final concentration of each to 10^{-3} M. (Substrate additions accounted for less than 5% of the final volume.) The volume was brought to 8 ml with TSB and was filter sterilized by passage through a 0.22-μm pore size membrane filter in a microsyringe filter holder. After filtration, 2.0 ml of sterile horse serum was added. The vial was loosely capped and placed on a reciprocating shaker at room temperature overnight. This latter procedure was necessary to reduce levels of dissolved $^{14}CO_2$ in the medium. Sterile medium was stored at −5 C.

Apparatus. A radiorespirometer was constructed (Fig. 1). The design was such that: (i) membrane filters (25-mm diameter) would lie flat in a 0.1-ml volume of medium; (ii) $^{14}CO_2$-collecting pads containing aqueous $Ba(OH)_2$ could be changed at intervals without jeopardizing the sterility of the system; (iii) a tight-fitting, closed system prevented evaporation of the medium and would provide for the possible future assay of anaerobes; (iv) the entire unit was autoclavable; (v) inside heat space volume was small to promote rapid diffusion of evolved $^{14}CO_2$ and minimize the loss of $^{14}CO_2$ by adsorption on the walls of the apparatus.

The culture cups were separated from the connector and autoclaved in petri dishes. The connectors were wrapped in foil, autoclaved, and attached to a ring stand by clamps just prior to the start of an experiment.

Detection procedures. Bacterial suspensions were filtered directly or added to human blood and lysing solution and filtered through a membrane filter. The filter was then transferred to the incubation cup containing 0.1 ml of radioisotopically labeled medium. The cup was immediately attached to the radiorespirometer, and $^{14}CO_2$ collection was initiated by placing an adsorbant pad in the collection cup and moistening it with one drop of saturated $Ba(OH)_2$ solution. The $Ba(OH)_2$ pads were changed at intervals, the exposed pads were dried, and the radioactivity was determined in a gas flow counter (Nuclear-Chicago Corp., model 1040).

Aliquots of sterile TSB were also filtered, the membrane filters were placed in the radiorespirometers with ^{14}C-labeled medium, and the evolved $^{14}CO_2$ was collected. Results from this sterile medium con-

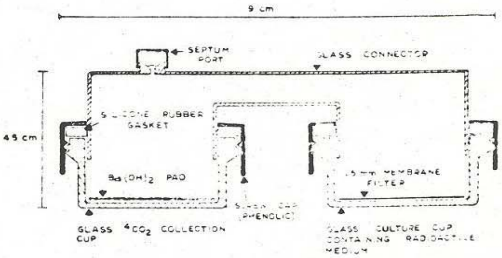

FIG. 1. *Diagram of radiorespirometer.*

stituted the control. A series of controls was conducted on each medium batch. Averages and standard deviations (σ) were determined for the controls for each time interval of incubation. To be classified as positive, a test level exceeding the average control by three σ or more was required. Different batches of ^{14}C-labeled medium displayed slightly different control levels; therefore, it was necessary to prepare a three σ curve for each batch.

RESULTS

Detection of bacteria. Incubation times producing positive signals for dilutions of various bacteria are presented in Table 1. Ten of 13 organisms tested gave a positive signal from approximately 10 CFU in less than 2 h, a significant improvement in rapidity and sensitivity of response over results reported heretofore. Higher cell concentrations were detected sooner than lower cell concentrations. Some organisms were positively detected by early readings, but produced signals below the positive level during continued incubation. Generally these organisms reverted to positive responses again later during the incubation. As

TABLE 1. *Detection times for various bacteria*

Organisms and CFU added/filter	Detection time (h)[a]												CFU recovered[b]				
	1	2	3	4	5	6	7	8	9	10	11	12	22	10	100	1,000	10,000
E. coli																	
10		−		−		−		+		+		+	+	6			
100		−		+		+		+		+		+	+		32		
1,000		−		+		+		+		+		+	+			TNTC[c]	TNTC
10,000		+		+		+		+		+		+	+				
P. aeruginosa																	
10		−		−		−		+		+		+	+	11			
100–10,000		+		+		+		+		+		+	+		TNTC	TNTC	TNTC
Klebsiella sp.																	
10	+		−		−		+		+				+	10			
100–10,000	+		+		+		+		+				+		44	TNTC	TNTC
S. aureus																	
10–10,000		+		+		+		+		+	+	+	+	15	52	TNTC	TNTC
Streptococcus (α hemolytic)																	
10	−	+		−		+		+		+		+	+	3			
100	−	+		+		+		+		+		+	+		5	9	45
1,000–10,000	+	+		+		+		+		+		+	+				
S. paratyphi B																	
10–10,000	+	+		+		+		+		+			+	7	52	TNTC	TNTC
S. typhi																	
10–10,000	+	−		+		+		−		+			+	—	—	—	—
S. dysenterae																	
10–10,000	+	+	+		+		−		−		+		−	2	2	13	TNTC
H. aphrophilus																	
10		−	−		−								−	9			
100–10,000	+	+	+		+		−		−				−		100	TNTC	TNTC
E. aerogenes																	
10–10,000	+	+	+		+		−		−				−	10	TNTC	TNTC	TNTC
S. marcescens																	
10	−	−	−		−				+		+		+	0			
100	−	−	+		+		+		+		+		+		100		
1,000–10,000	+	+	+		+		+		+		+		+			TNTC	TNTC
P. vulgaris																	
10–10,000	+	+		+		+		−					+	0	0	3	TNTC
Cardiobacterium sp.																	
10	+	+	+		+		+				+		+	0			
100	+	+	+		+		+				−		−		0		
1,000	−	+	+		+		+				+		+			0	
10,000	+	+	+		+		+				+		+				3

[a] +, 3 σ above average control; −, less than 3 σ above average control.

[b] After 24-h incubation in radiorespirometer, filters were aseptically transferred to an agar plate and incubated an additional 24 h.

[c] TNTC, Too numerous to count.

seen in Table 1, hemolytic *Streptococcus*, *H. aphrophilus*, *Klebsiella* sp., and *Cardiobacterium* showed this phenomenon. However, *Cardiobacterium* (100 CFU) which was positive at early times fell below the three σ level during continued incubation. Figures 2 and 3 show the kinetics of $^{14}CO_2$ evolution by approximately 100 CFU of bacteria in Table 1. Bacteria produce an initial curve which is parallel to, but higher than, the control curve. For most organisms, the curve breaks away from the control curve and rises sharply. In some cases, however, the bacterial curve remains at a low (even though positive) level throughout the 22-h incubation period. It appears that cells produce a small but detectable "early burst" of $^{14}CO_2$ initially. Most organisms then undergo a period of adaptation, of varying duration, and finally growth which results in the generation of considerable $^{14}CO_2$. Some organisms such as *Cardiobacterium* and *S. paratyphi* B apparently did not adapt and grow within the 22-h incubation period.

Some organisms, on the other hand, produced much $^{14}CO_2$ but were not recovered or showed poor recovery. This phenomenon appears to be due to inhibition of growth by membrane filters.

Detection of bacteria in blood. The detection of bacteria in blood by the respirometric method requires that the blood be sufficiently lysed to allow filtration through a membrane filter and to preclude a false-positive response generated by intact blood cells. However, the technique must not be damaging to bacteria. A lysing method based upon that reported by Rose and Bradley (17) was used during initial studies. Bacteria were inoculated into blood, which was then lysed, filtered, and monitored for evolution of $^{14}CO_2$. Controls were blood alone. A ratio of counts per minute evolved by the inoculated blood to the counts per minute evolved by uninoculated blood was obtained at various time intervals. Results (Fig. 4) show individual curves obtained in a number of experiments by using different media batches and blood samples. Although a threshold control level for normal blood cannot be represented, $^{14}CO_2$ evolution from low numbers of bacteria added to blood can be clearly distinguished from $^{14}CO_2$ evolution by blood cells. The method allowed low numbers of bacteria in blood to be detected within a few hours; however, adverse effects of the lysing solution on bacteria were observed. Bacterial numbers in the inoculum were verified by plate counts, and the recovery of inoculated bacteria was checked by counting colonies which occurred on the test filters. Recovery of gram-positive bacteria was generally

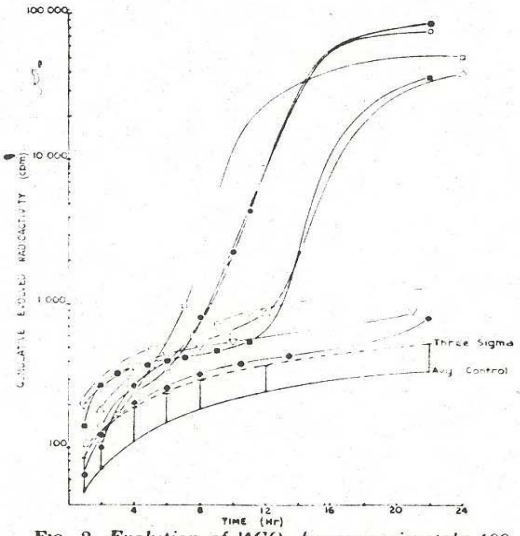

FIG. 2. *Evolution of $^{14}CO_2$ by approximately 100 CFU of: ●, E. coli; ○, P. aeruginosa; □, Klebsiella sp.; ■, S. typhi; △, S. aureus; ◇, S. dysenteriae; ▽, S. paratyphi B; ◆, alpha hemolytic Streptococcus. The average control level and 3 σ confidence limit are given for medium alone.*

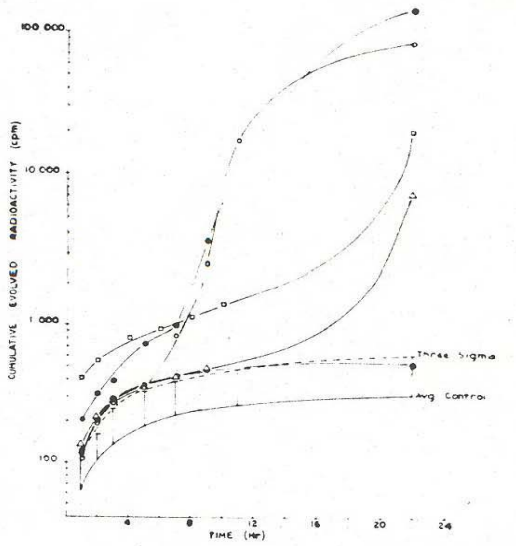

FIG. 3. *Evolution of $^{14}CO_2$ by approximately 100 CFU of: ●, Enterobacter sp; ○, S. marcescens; □, P. vulgaris; △, H. aphrophilus; and ◆, Cardiobacterium sp. The average control level and 3 σ confidence limit are given for medium alone.*

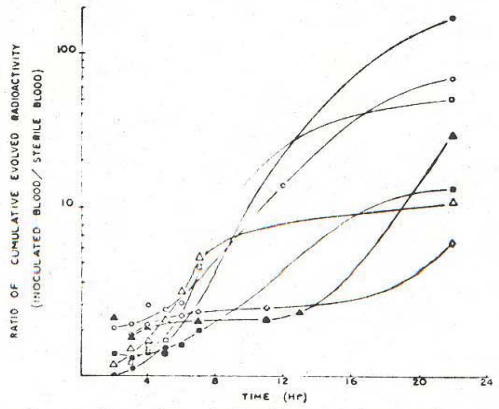

FIG. 4. *Detection of:* ●, *P. vulgaris (7 CFU);* ○, *pneumococcus (26 CFU);* □, *E. coli (10 CFU);* ▲, *Candida albicans (10 CFU);* ■, *P. aeruginosa (45 CFU);* △, *S. aureus (120 CFU); and* ◇, *enterococcus (15 CFU) in blood. Inocula were added to 1 ml of blood which was then lysed by the method of Rose and Bradley (17) and filtered.*

better than gram-negative bacteria; however, the evolution of $^{14}CO_2$ from both types was found to be decreased by the lysing agent. Farmer and Komorowski (4) also reported poor recoveries of some bacteria. Most bacteria which showed poor recovery also showed a decreased 22-h cumulative $^{14}CO_2$ evolution. However, the $^{14}CO_2$ evolution during the first 7 h was generally slightly greater for the bacteria which were inoculated into blood and then lysed than for bacteria alone.

Attempts were made to use a lysing solution less detrimental to bacterial cells than the highly alkaline 0.05% Triton X solution. The protease Rhozyme in conjunction with an aqueous solution of Triton-100 was found to be an effective, noninhibitory lysing solution. Table 2 shows the results of pure culture bacteria recovery after treatment with Rhozyme and Triton X-100. Recovery was approximately 100% with all organisms tested even in the presence of 10-fold higher concentrations of the lysing agents. Some organisms actually proliferated in the lysing solution during the 30-min incubation period.

Various final concentrations of Rhozyme (1.93 to 10 mg/liter) and Triton X-100 (0.036 to 0.3 mg/ml) were tested to determine the filterability of 4 ml of blood so treated. Filterability through a 0.65-μm pore size, 25-mm diameter membrane filter was measured. The less filterable mixtures filtered rapidly initially; but, as the filter clogged, the rate of filtration markedly decreased. Increased concentrations of either Rhozyme or Triton X-100 improved filtration. However, the Triton X-100 appeared to be a far more critical reagent. The highest concentrations of Rhozyme and Triton X-100 investigated (see Table 2) produced a filterable mixture after less than 15 min of incubation.

Several experiments were conducted to determine if bacteria may pass through the 0.65-μm pore size filter. Although very small organisms were not investigated, there was no significant difference in the number of CFU occurring on 0.45- and 0.65-μm filters used to filter bacterial suspensions of *E. coli*, *P. aeruginosa*, *S. marcescens*, and *S. aureus*.

Figure 5 shows typical results which were obtained for low numbers of *E. coli* in blood. Note that blood plus inoculum produced a

TABLE 2. *Recovery of bacteria treated with Rhozyme and Triton X-100*

Bacterium	Total vol (ml)[a]	Rhozyme (mg/ml)	Triton X-100 (mg/ml)	% Recovery
E. coli	37.5	2.13	0.040	116
E. coli	39.0	2.05	0.077	89
S. aureus	39.0	2.05	0.077	154
P. vulgaris	39.0	2.05	0.077	88
P. aeruginosa	39.0	2.05	0.077	95
E. coli	42.5	2.75	0.103	100
S. aureus	42.5	2.75	0.103	120
P. vulgaris	11.0	10.90	0.409	150
P. aeruginosa	11.0	10.90	0.409	93

[a] Volume of lysing solution only. No blood was used in these experiments.

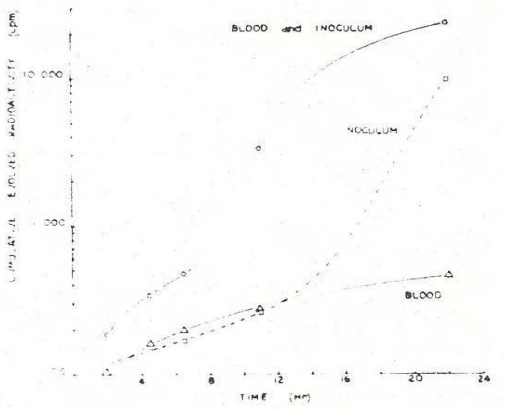

FIG. 5. *Detection of approximately 13 CFU of E. coli which were added to 4 ml of normal blood and lysed with Rhozyme and Triton X-100. Data shown are the average of duplicate determinations.*

greater and more rapid evolution than the same amount of inoculum alone. These results suggest that material from the blood which remains on the filter in some way enhances growth.

DISCUSSION

The procedure for the rapid radiorespirometric detection of bacteria in blood is shown schematically in Fig. 6.

The evolution of radioactivity from a medium containing ^{14}C-labeled compounds, by low numbers of bacteria, appears to follow a pattern which consists of an early evolution of a small but detectable amount of $^{14}CO_2$ evolution followed by a lag phase of several hours. Then a rapid evolution of $^{14}CO_2$ begins and produces a high cumulative level of evolved radioactivity. To detect the presence of microorganisms rapidly, it is necessary either to use a system which is sensitive enough to distinguish the early burst from control levels or to establish conditions which decrease the lag phase. The former approach saves valuable time and also permits detection of some organisms which do not adapt to the medium and fail to produce the characteristic rapid evolution of radioactivity after the early burst.

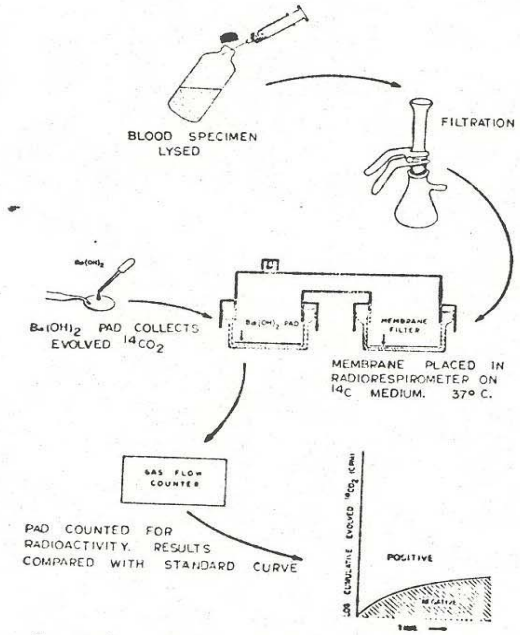

FIG. 6. *Procedure for rapid radiorespirometric detection of bacteria in blood.*

Of the two methods for lysing blood cells, 0.05% Triton X-100 plus 0.8% Na_2CO_3 and Rhozyme plus 0.005 Triton X-100, the latter appeared to be far superior. The Rhozyme-Triton X-100 method was not toxic to the bacteria tested; and, in some cases, growth actually occurred in the lysing solution.

The method herein described includes the advantage of both the membrane filter culture technique and rapid detection by radiorespiration. Lysis and filtration of blood as described concentrate bacteria and presumably wash them free of inhibitory blood constituents and antibiotics. In some cases, filtration may also provide a larger microbial sample, e.g., sterility testing of air of filterable liquids.

The use of a small amount of ^{14}C-labeled medium produces an earlier response than methods involving larger volumes of medium (1, 2, 3, 16, 20, 21). This large inoculum rapidly poises the medium and initiates growth. Lowering the pH by the bacteria as well as the large surface-volume ratio also facilitates rapid evolution of $^{14}CO_2$.

The use of a small volume (0.1 ml) of ^{14}C-labeled medium allows for the economical use of relatively high levels of radioactivity and expensive nonradioactive enrichments. The high level of radioactivity greatly increases the sentivity of the assay. Lysis and filtration eliminate much of the radiorespirometric response caused by blood cells, thereby increasing the sensitivity of the radiorespirometric detection.

Extended incubation of filters which show positive radiorespirometric results provides isolated colonies in the shortest possible time and with no additional manipulation, which may be used for sensitivity and identification determinations. The number of colonies on a filter also provides a quantitative estimate of bacterial numbers in the sample, and, in some cases, may assist in differentiating contamination from bacteremia.

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