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Rapid Microbiological Determinations with Radioisotopes

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I. Classic Microbiological Techniques

A. GENERAL TECHNIQUES

The microscope had been known for nearly four centuries when Leeuwenhoek made his startling discovery of bacteria in 1676. Ever since this historic event, direct observation has been one of the principal methods by which. microorganisms have been detected, identified, and studied. For nearly another hundred years, this was the sole method available. Then Pasteur conducted his brilliant experiments. In proving that fermentation was caused by bacteria, he simultaneously provided a powerful new tool for bacteriological determinations—the culturing of bacteria in nutrient media. Masses of bacteria and the effects produced by them on inoculated materials could be observed.

The two techniques, microscopy and culture, thus provided means for the micro and macro study of organisms invisible to the naked eye. The introduction of staining by Weigert, Ehrlich, and Salomonsen helped bacteriological microscopy to reach its present state of attainment. When mechanical improvements refined the microscope to the limit imposed by optical resolution, the invention of the electron microscope greatly increased useful magnification. Correspondingly, the development of transparent, solid media by Koch in 1881 implemented the development of the colony technique. The introduction of selective media further enhanced the usefulness of culturing. More recently, serological and enzymatic reactions have offered important new methods for the study of microorganisms.

B. TIME FACTOR

The identification and enumeration of microorganisms by these now classic techniques are determined by: inspection of the cells, inspection of colonies, the development of turbidity, the formation of gas bubbles, color changes or other physical changes in the reaction mixture. Despite the wide diversity of these criteria, they have one aspect in common: all are of a direct visual nature. As a consequence, the quantitative examination of an unknown sample by these methods consumes considerable time.

Microscopic inspection permits rapid identification of morphological types, but different species with the same morphology cannot be distinguished. The enumeration of a representative sample is exceedingly tedious, especially where small numbers of bacteria are concerned, because statistical confidence requires the time-consuming observation of a great many fields. Dilution and plating techniques permit quantitative determinations by the colony method. However, since each cell must give rise to a visible colony, time must be allowed for the reproduction of many generations. The same incubation time requirement confronts quantitative application of the gas bubble and turbidity methods in liquid media. On the other hand, enzymatic reactions can produce color or other changes in the reaction mixture rapidly. Their use, however, has been limited to the examination of materials, principally milk, in which relatively large numbers of bacteria are normally found. The method is inherently very sensitive and its sensitivity can be greatly extended by fluorescent techniques and the use of photomultiplier apparatus (Laurence, 1957). Although enzymes generally react with specific substrates, the various enzymes are so widespread in nature that the use of this technique for the identification of specific microorganisms seems generally precluded. Serological reactions are specific, but, as used, require large numbers of cells.

As a consequence of the above considerations, all standard methods used for the quantitative identification of small numbers of cells of a particular species or group of organisms in unknown samples require from 24 hours to several days for completion.

1. *Significance of Delay*

For most research studies, this time delay is inconvenient, but seldom constitutes a serious problem. In the fields of medicine and public health, however, the matter of time is frequently paramount. In monitoring a product or an environment for bacteria, even a one day period! of ignorance may constitute a serious hazard.

a. Water Supplies. The bacteriological control of public water supplies is an outstanding example in this category. The established (Public Health Serv., 1962) index for the bacteriological quality of drinking water is the coliform organism group. These organisms live in the intestinal tracts of warm-blooded animals and are, consequently, present in great numbers in sewage. They are discharged in such quantities that, even when sewage is diluted to the point where the receiving water is aesthetically acceptable, the presence of the coliform organisms can readily be established. Although most of the bacteria in the coliform group are not pathogenic, their demonstrated presence is grounds for rejection of the water for drinking purposes on the assumption that pathogenic organisms are also present.

The most widely used standard method (Am. Public Health Assoc., 1962) for the quantitative determination of coliform organisms is the most probable number technique based on serial dilution of the sample. The quantitative aspect of this test relies upon the isolation of a single cell in a diluted aliquot of the sample. Such aliquots are incubated in lactose broth where the production of gas constitutes positive evidence. To produce a 1 mm. diameter bubble, a population of 1.7×10^9 cells must result from the single bacterium. Forty-eight hours must be permitted to elapse before the test can be presumed to be negative. Should the test become positive after either 24 or 48 hours, a transfer must be made into a more selective medium for confirmation. Confirmation requires another 48 hours before negative results can be accepted, although the tubes may produce gas for a positive result after 24 hours. Thus, a minimum of 48 hours must elapse for a positive determination, and a period of 72 or 96 hours must elapse for a negative sample which gave a positive presumptive test. As a result, in most municipalities, the water is consumed by the public before the bacteriological quality is ascertained. Although careful process control safeguards the water, this ignorance of the principal criterion of potability has resulted in disease outbreaks.

Recently, a second standard method (Am. Public Health Assoc., 1962) for the quantitative determination of coliform organisms in water was adopted. This method uses a submicron filter through which the sample is drawn. The "membrane" filter is then placed on a pad saturated with a coliform group selective medium which rises through the pores of the filter and permits the supposedly isolated cells on the filter to develop into visible colonies. Twenty ± two hours of incubation are required for the completion of this test. Even this delay relegates bacteriological results to the realm of historical information in most municipalities.

Rapid bacteriological determinations in water supply quality control would also be helpful in determining raw water quality. Such information would assist in intelligent process control, and, in event of gross contamination, the source could be rejected. Here again, the time required by the standard bacteriological methods prevents use of bacteriological data except in retrospect.

b. Swimming Pools. At swimming pools and natural bathing areas, the time delay in obtaining bacteriological results also creates a public health problem. Water quality control based on bacteriological results is impossible. Nonetheless, the primary criterion for bathing waters is the bacteriological one. Here, the need is not only for a rapid method, but for a simple one which can be administered by the pool or beach operator. Otherwise, inspectors from the health department must transport samples back to the central laboratory for determinations. Such visits can at best be only infrequent with respect to the public exposure time.

The ideal method for meeting the public health requirements for determinative bacteriology at water treatment plants and swimming pools would be a periodic sampler and analyzer which would obtain analytical results rapidly enough to operate feedback mechanisms controlling the water production process. Specifically, the coliform organism level might be used as a direct control of the level of chlorination. Until such a method is available, actual reliance on bacteriological quality control will continue to be careful process operation, particularly the maintenance of an adequate chlorine residual in the water. With modern treatment methods, this is normally satisfactory. However, if this were a completely reliable safeguard, the public health standards would be couched in terms of chlorine residuals, which can be determined immediately, rather than in terms of bacteria.

c. Food. Another important area of public health bacteriology is that of food processing and serving. The packaging of sea food, dairy products, vegetables, and poultry would benefit from a bacteriological method rapid enough to permit early measurement of the quality of the raw foods and the quality maintained through the various process steps. The packaging of unpasteurized, frozen foods has greatly increased this need in recent years. Moreover, frozen foods should be bacteriologically analyzed in storage and on display. Freezer power failures or improper temperature control frequently place food which has been defrosted a number of times in the hands of the consumer.

The problem in food serving establishments is much like that of the swimming pool, where periodic—or sporadic—sampling of food and utensils by health department inspectors supplies information suitable only for identifying habitual offenders.

d. Selection of Antibiotics. A second major field requiring rapid bacteriological methods is medicine. In many instances, rapid identification of bacterial infection would make treatment more effective and could even save lives. If the infectious organism could be identified rapidly, this would permit selection of the preferred chemotherapeutic agent. However, a method which would not identify the organism, but would determine the treatment agent of choice would be equally effective. Either method would have benefits beyond those associated with treating the particular infection. Because such knowledge is not readily available in time for its effective use, broad spectrum chemotherapeutic agents are frequently used. Many times, knowledge of the infection would indicate against such use. Administration of these agents sometimes sensitizes the patient, resulting in considerable hazard being associated with his future use of the agent. Furthermore, widespread use of some antibiotics has rendered them less effective by promoting the selection of resistant strains of organisms. The elimination of nonessential use of antibiotics would alleviate this problem in many instances.

e. Bacteriological Warfare. There is, regrettably, a third major demand for rapid bacteriology. This is the requirement for adequate defense against bacteriological warfare. Before appropriate measures can be taken to protect populations, the attack must be detected. Means for delivering BW agents through air or water have become sophisticated to the point where there may be no overt indications of an attack. Only through detecting the bacteria themselves can knowledge of such an attack be ascertained reliably. Only the briefest time, perhaps several minutes, will be available for protective measures. Thus, the attack must be detected almost immediately. This formidable problem has been approached along several avenues. Identification of the specific pathogen would require perfection of a rapid test for each of the species suitable for bacteriological warfare. With less difficulty, an alarm might be based upon the detection of a rapid rise in the background count of microscopic particles. Dust or other particles could produce false alarms with such a system. Chemical identification of the media in which the bacteria were grown or transported might be used as an index. However, such analyses would still not prove the presence of living organisms. While much of the information on BW defense is classified, published accounts indicate that the two principal approaches are the development of rapid particle size analyzers which will signal an alarm when the background levels significantly change, and the development of a device which stains and microscopically detects living particles. Although current state of the technique must remain obscure for security reasons, the Army Chemical Corps has frequently and publicly announced its urgent need of improved BW detection methods.

II. Radioisotope Technique

A. BASIC CONSIDERATIONS

To eliminate the growth period in a quantitative bacteriological determination requires a method with a resolving power at least as great as that permitted by visible light, and a means for the rapid examination of a statistically significant portion of the unknown sample. The two requirements tend to be mutually exclusive. A practical consideration adds to the problem: the method or instrument must be simple enough to serve as a routine laboratory tool.

The great jump in analytical sensitivity provided by the introduction of radioisotope techniques and a fortuitous aspect of biochemistry combine to make the desired test possible. The increased sensitivity offered can be appreciated by the fact that radiation detection instruments can detect a beta particle ejected from an atomic nucleus. The beta particle is many trillions of times smaller than a bacterium. The physical elements of the technique are thus satisfied. The remaining requirement is the biochemical one—to achieve the desired selectivity in applying the method to determinative microbiology. The simplest approach is to rely on the selectivity of existing tests by using the same media and conditions, the only innovation being appropriate labels. This is possible where the procedure permits only the organisms of interest to grow. The problem is more complicated with organisms that are identified by color or sheen developed in media which also permit growth of other organisms. In such cases, new criteria must be applied based on other distinguishing characteristics of the species. Some of these characteristics might, themselves, be determined through the use of isotopes. It is conceivable that a new array of selective, radioactive media could be developed in much the same manner as was the present arsenal of the microbiologist.

That bacteria could be induced to incorporate substrates containing radioactive atoms which could then be followed to elucidate metabolic pathways has been demonstrated by Cowie *et al.* (1950, 1951, 1952a, b) and in the extensive work of Roberts *et al.* (1955). Massive quantities of bacteria were used in these studies and the principal method of determining the disposition of the radioactive atoms was by radioautography of chromatograms.

A practical bacteriological test using isotopes requires that the radioisotope be easily introduced and easily recovered from the bacteria or metabolic products. If the bacteria or the metabolic products retained in the medium are to be sought as evidence, the problem becomes difficult. This is because a physical separation of the unused, labeled substrate from the bacteria or metabolic products would have to be accomplished before results could be obtained since the isotope detection equipment cannot distinguish any one of these fractions from the others. Moreover, the separation is complicated by the fact that, after a brief exposure to the bacteria, most of the label remains in the unused substrate. Therefore, unless separation is complete, traces of the substrate will mask the presence of the organisms and the products produced. Such separation from the medium would be extremely difficult to accomplish with the desired rapidity.

The fortuitous circumstance making the isotope method readily applicable is the fact that a substantial portion of carbohydrate carbon taken in by cells utilizing the Krebs cycle is oxidatively metabolized to carbon dioxide. Thus, converted to a gas, metabolized radioactive carbon readily separates from the liquid culture medium for easy collecting and counting. The same advantages, of course, hold for other isotopes producing other gases. The technique implied by these facts is sensitive enough to detect the respiration of small numbers of resting cells in a matter of several minutes or hours.

B. APPLICATIONS

1. *Coliform Test*

The first goal of the radioisotope technique was a rapid test for the coliform group of organisms. The standard method (Am. Public Health Assoc., 1962) multiple tube fermentation test offered the possibility of direct adaptation. This is the test in which the fermentation of lactose with the production of gas constitutes a positive finding. The test is generally applied in two steps, one presumptive and the other confirmatory. The tube portions positive in the presumptive test are transferred to tubes of lactose broth containing dyes inhibitory to noncoliform organisms. Production of gas in the latter tubes confirms the test. Approximately one-third of the gas produced by coliform organisms is carbon dioxide. Appropriate labeling of the lactose with C^{14} results in the production of $C^{14}O_2$. The $C^{14}O_2$ can be captured readily with barium hydroxide or other "getters." The radioactivity collected on the getter can then be measured and is an index of the metabolic activity in the sample.

As the method was first reported (Levin *et al.*, 1956), a portion of a water sample in question was inoculated directly into 10 ml. of lactose broth in which the 0.5% lactose content was supplied with lactose-1-C^{14} synthesized by Frush and Isbell (1953). The apparatus consisted of a train through which filtered air was bubbled into the inoculated culture. The air entrained $C^{14}O_2$ produced by the culture and carried it through a vapor trap, to reduce possible aerosol carry-over, and finally through a porous paper pad impregnated with several drops of a saturated solution of barium hydroxide. At suitable intervals, the pad was replaced and the exposed one dried and counted in an internal flow counter. This process paralleled the standard presumptive test for coliform organisms, merely substituting a more sensitive method for the detection of the gas evolved. The sensitivity achieved is demonstrated in Table I. As few as 125 cells were detected in 1 hour. The principal drawback of this approach was its high cost imposed by the large quantity of isotope used. In the course of development (Levin *et al.*, 1957, 1961), this problem has been met by reducing the quantity of isotope required and by substituting the much less expensively prepared formate-C^{14} for the lactose-1-C^{14}. The possibility of using formate was indicated by the standard (Am. Public Health Assoc., 1962) formate ricinoleate broth and by the determination by Roberts *et al.* (1955, p. 166) that 86% of the carbon utilized by *Escherichia coli* as formate was converted to CO_2. While the incidental developmental details can be obtained from the references cited, it is felt that a description of the method in its current form may be worthwhile.

TABLE I[a]
PRESUMPTIVE TEST OF SAMPLE CONTAINING APPROXIMATELY 125 *E. coli*

Time (hour)	Radioactivity of test[b] (counts per minute) Increment	Radioactivity of test[b] (counts per minute) Cumulative	Radioactivity of control[b] (counts per minute) Increment	Radioactivity of control[b] (counts per minute) Cumulative
1	172	172[c]	67	67
2	309	481	38	105
3	1,154	1,635	36	141
4	4,075	5,710	36	177
5	12,579	18,289	27	204

[a] From Levin *et al.* Reproduced courtesy *J. Am. Water Works Assoc.* **48**, 1, 77 (1956).

[b] Radioactivity measured above a background of 21 counts per minute.

[c] Point of presumptive determination.

The apparatus consists of a commercially available membrane filter assembly, membrane filters, and paper absorbent pads, all of one-inch diameter; a vacuum pump or aspirator; a shaker; aluminum planchets one inch in diameter by one-fourth inch deep with a flat lip one-eighth inch wide; 35 mm. by 50 mm. glass cover slips; calibrated pipettes; a hot plate or heat lamp; and a commercially available end window or gas flow radiation detector with associated scaler. Most of these items are shown in Fig. 1.

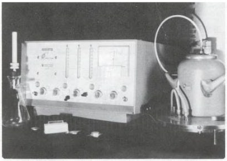


Fig. 1. Apparatus for rapid coliform organism test. Left to right (*foreground*): membrane filter apparatus, $C^{14}O_2$ collection planchet, cover slips, culture planchet with cover slip in place, assembled culture-collection unit; (*background*): scaler, internal flow counter.

The method is a one-step, confirmed test for fecal coliform organisms. Narrowing of the coliform group to those coliforms of fecal origin increases the sanitary significance of the test. British MF MacConkey broth (Membrane Filtration) to which sodium formate-C^{14} is added is the medium used. The ingredients are: 3% lactose, 1% peptone, 1% bile salts, 0.5% NaCl, 0.0012% brom cresol purple; 0.002% sodium formate-C^{14} (8 mc./millimole). Sterilization of the medium is accomplished by autoclaving for 15 minutes at 15 p.s.i. or by membrane filtration. The flask containing the medium is then stoppered with sterile cotton and shaken for several hours or overnight to reduce, by atmospheric exchange, small amounts of non-metabolic $C^{14}O_2$ generated in the sterile medium.

The desired quantity of the water sample is drawn through a filter membrane. The membrane is aseptically placed into a sterile planchet. Then, 0.5 ml. of the medium is pipetted onto the membrane and a cover slip is immediately placed over the planchet.

The oxygen restriction thus enforced increases the specificity of the test. Together with a sterile control, the test portion or portions are incubated at 44°C. After 3½ hours, the planchets are removed from the incubator. A tightly fitting paper pad is pressed into the bottom of each of an equal number of planchets. Five drops of a settled, saturated solution of barium hydroxide are then delivered onto each pad. The planchets containing the pads are quickly inverted on the cover slips of the culture planchets. The cover slips are slid out from between the planchets which then enter into direct communication with each other. Carbon dioxide evolved from the culture planchet will leave the broth and travel to the absorbent pad under the impetus of the concentration gradient created by the fixation of the gas on the pad in the form of barium carbonate. Immediately after being united, the paired planchets are returned to the incubator for 30 minutes, providing a total incubation period of 4 hours. The planchets are then removed from the incubator and the pairs separated. Those planchets containing the pads are placed on a hot plate or under a heat lamp for several minutes. Still in their original planchets, the dried pads are counted for radioactivity. Counting to a satisfactory degree of significance can generally be achieved within several minutes.

Because the use of the membrane filter has been shown (Levin *et al.*, 1961) to reduce markedly the sensitivity of the test, the ultimate sensitivity is best demonstrated by showing data obtained using this method with the exception that the inocula were applied by pipetting 0.1 ml. portions of test suspensions rather than by filtration. Table II lists values obtained with *E. coli* ATCC 8739. Each value is an average of 5 replicates with background and sterile control levels subtracted. The numbers of cells producing the responses were determined by nutrient agar pour plates.

TABLE II[a]
RESULTS OF 4-HOUR RADIOISOTOPE TEST ON *E. coli* ATCC 8739 USING NONFILTERED INOCULA AND MF MacCONKEY BROTH

Inoculum (no. cells)	Average counts per minute	Counts per minute per initial cell[b]
12	57	4.75
28	263	9.40
77	625	8.12
83	807	9.73
85	391	4.60
975	7,120	7.30
1,170	5,540	4.73
2,460	16,600	6.75
9,820	70,600	7.19
41,600	211,000	5.08

[a] From Levin *et al.* Reproduced courtesy *J. Water Pollution Control Federation* **33**, 10, 1024 (1961).

[b] Average counts per minute per cell = 6.77.

When several commercial types of filter membranes were used, the $C^{14}O_2$ produced in the 4-hour period was approximately one-tenth of that evolved by equal inocula applied by the pipette method. Figure 2 illustrates this effect. One type of filter membrane, Gelman Type 27A, was found to produce only a twofold reduction in $C^{14}O_2$ production. Figure 3 compares equal inocula applied by filtration and pipetting. Quantitative data on fecal coliforms in water samples have been collected using this filter, but have not yet been published. By way of interest to the quantitative aspect, Table III shows the relationship between various ranges of cell populations and the 4-hour responses obtained by an earlier version (Levin *et al.*, 1959) of the test.

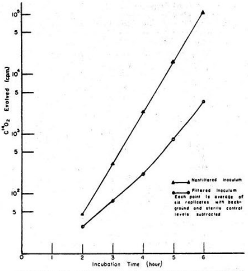


FIG. 2. Effect of membrane filter on *E. coli* ATCC 8739. $C^{14}O_2$ evolved as a function of time for filtered and nonfiltered equal inocula. Almost perfectly straight line of nonfiltered inoculum curve demonstrates accuracy of gas production as index of growth. From Levin *et al.* Reproduced courtesy of *J. Water Pollution Control Federation* 33, 10, 1026 (1961).

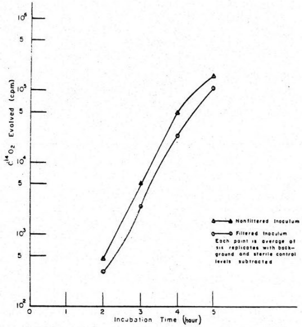


FIG. 3. Effect of Gelman Type 27A membrane filter on *E. coli* ATCC 8739. $C^{14}O_2$ evolved as a function of time for filtered and nonfiltered equal inocula. From Levin *et al.* Reproduced courtesy of *J. Water Pollution Control Federation* 33, 10, 1035 (1961).

TABLE III[a]

CORRELATION BETWEEN *E. coli* POPULATION AND EVOLVED RADIOACTIVITY

Run no.	Evolved radioactivity counts per minute[b] 1–25 Cells	25–50 Cells	50–100 Cells	100–200 Cells	200–400 Cells	400–800 Cells	800–1600 Cells	1600–3200 Cells	3200–6400 Cells
1[c]		27	64	94	181	400	792	1625	4290
2	7		31		106				
3	11	37	89	155	224	449	906	1820	6271
4		37	52	135	391	673	1373	2283	6060
5		24	39	93	164	298	768	1336	
6	16	24	57	96	224	472	858	1621	
7	15	28	40	105	245	755	1676	4000	6262
8			14	36	109	254	669		
9		15	37	66	167	321	771	1871	
10					117	247	520		
11					149	285	480		
12					358	488	985		
13						342	762	2400	5450
Average	12	27	47	98	203	415	880	2119	5667
Standard deviation	4	7	21	37	92	163	340	841	839

[a] From Levin *et al.* Reproduced courtesy *Journal Am. Water Works Assoc.* **51**, 1 (1959).

[b] Average of three replicates without sterile controls.

[c] Blanks indicate no data available.

a. Factors Affecting Accuracy of Standard and Rapid Tests. The radioisotope coliform test has some advantages beyond those cited above. Although replicate portions have routinely been used in testing samples, these are for the purpose of increasing statistical reliability rather than, as in the case of the multiple tube dilution test, to satisfy the requirement for a quantitative determination. As its name implies, the multiple tube technique requires that replicate portions (generally five) of several dilutions of the sample (generally three) be inoculated to permit the determination of the most probable number of coliform organisms in the original sample. The comparative simplicity of the rapid test makes it convenient to test several replicates of each sample. In effect, each replicate is equivalent to one complete set of dilution tubes in the most probable number technique. Fundamental to the statistical approach of the multiple tube method is the assumption that the gas bubbles in each of the highest dilutions found to be positive originated from a single cell inoculum. As will be discussed, this assumption is not valid. Accuracy of the multiple tube dilution test also suffers from the fact that a substantial number of false positives frequently results, even through the confirmed step. A third principal source of error is introduced by statistical effects and bias in the quantitative determination as shown by McCarthy *et al.* (1958) and McCarthy (1961).

Quantitative results with the membrane filter test are also subject to error (Levin *et al.*, 1961; McCarthy, 1961). Some of the difficulty may arise from toxic manifestations with some types of filter membranes (Levin *et al.*, 1961). The numerical aspect of this test likewise depends upon isolation of single organisms. Jones and Jannasch (1956) have shown that, in reality, a high percentage, probably the majority, of the cells exist and are deposited as clumps, hence giving rise to fewer colonies than the initial number of cells. Clumping does not operate against the radioisotope method since the quantitative aspect of the latter is not derived from direct visual evidence. The total quantity of gas produced by the organisms present constitutes the parameter measured and is probably not materially influenced by clumping.

The lack of the dilution requirement in the rapid test likewise serves to its advantage in a comparison with the membrane filter method. For statistical reliability in the membrane filter technique, it is recommended that the number of colonies developed be within the range of 20 to 200, preferably 20 to 80. Unless the approximate quality of the water to be tested is known in advance, several different quantities or dilutions of the original sample must be filtered and incubated to achieve this narrow range. Heavily polluted waters are frequently difficult, or even impossible, to test by the membrane filter. This is because noncoliform organisms, which greatly exceed the coliform organisms in the sample, also grow on the membrane filter and, while not exhibiting the identifying sheen of the coliform organisms, physically crowd out the latter. Sometimes, when the total organisms are sufficiently diluted, the coliforms are extinguished.

A further disadvantage of the dilution technique for either of the current standard methods is that dilution imposes nutrient, osmotic, temperature, and sometimes pH changes which result in the death of organisms. Finally, the radioactive method is sensitive enough to measure the respiration of "dead" organisms which do not achieve growth during incubation periods of the standard tests. Butkevich and Butkevich (1936) state that, at least in sea water, bacteria which do not respond to the usual media may constitute a significant fraction of the total organisms.

Having listed the advantages in accuracy that the radioisotope test enjoys over the current standard methods, it must now be said that the quantification of the rapid test has been one of its most difficult developmental problems. Much of the problem is a chicken or egg paradox. Against what can the sensitivity and the quantitative accuracy of the radioisotope method be calibrated? The sensitivity of the rapid test is greater than that of either standard method, and the quantitative accuracy of both standard methods has been shown to be considerably clouded. Because of the lack of an absolute standard, accurate calibration of the radioisotope test poses a quandary.

Replication by the rapid test is good when inocula of the same strain are compared within a single run, but not quite as good when different runs are compared. Good replication is also obtained with wild cultures within a single run, but considerable variation in counts per minute per cell is produced in different runs on wild cultures. Figure 2 demonstrates the excellent quantitative results obtained from *E. coli* ATCC 8739 within a single run as a function of time. The exquisitely straight line through more than four orders of magnitude plotted for the nonfiltered inoculum is in complete agreement with the theoretical exponential growth curve which would be expected under the test conditions. Returning to Table II, the counts per minute per initial *E. coli* ATCC 8739 cell are seen to range somewhat less than ±50% of the average. Considering the broad range of inoculum, 12 to 41,600 cells, the results are excellent as measured by current bacteriological standards.

When wild cultures obtained from surface waters were used, the range of counts per minute per cell for different runs extended to approximately one-half an order of magnitude on either side of the average. In these cases, calibration was made by the membrane filter method using British MF MacConkey broth. The question that so far has not been answered is how much of the variation is inherent in the radioisotope test and how much of it represents errors produced by the other methods. The error-inducing factors associated with the standard methods certainly implicate them. There are also potential sources of error characteristic of the radioisotope test. Among the various strains of coliform organisms tested in pure cultures, two have been found to differ in rate of CO_2 production by as much as an order of magnitude. The ranges of per cent abundances of the extreme strains in natural waters are not known so that the significance of the difference cannot be fully assessed.

Another source of error may be the immediate history of the wild cultures. Cells in lag phase have been found to produce considerably more CO_2 per capita than exponentially growing cells. This, however, may not be significant in that exponentially reproducing cells would be expected to occur in surface waters only under rare conditions and then in such quantities that their presence would be readily detected. Although MacConkey broth is believed to be highly specific for fecal coliform organisms (Taylor, 1959-1960), if present in sufficient numbers during the period while noncoliforms are being inhibited, the latter may produce detectable quantities of $C^{14}O_2$. The problem of the toxicity of the membrane filter introduces a common error into the radioactive and non-radioactive methods. Finally, results will differ if media of different specific activities are used. Care should be exercised in ordering the labeled compound and in mixing the medium. The half-life of carbon is sufficiently long so that no correction for shelf storage need be applied.

While additional research may correct some of the causes of variation in $C^{14}O_2$ production on a unit cell basis, a realistic appraisal of the tenfold variation discussed must conclude that this range of quantitative accuracy is as good as any, and better than most, conferred by currently accepted bacteriological techniques. Jannasch and Jones (1959) compared direct microscopic methods and culturing methods, including the membrane filter, on total bacteria in sea water. It was found that there were 13 to 9,700 times more bacteria by direct counts than by cultural methods. A mean of more than 125 times as many cells were found on membrane filters by microscopic counting than by subsequent counting of visible colonies.

The interests of absolute accuracy might be served by calibrating the radioisotope test by means of micromanipulation of one or several cells. Such a technique might permit accurate knowledge of the size of the inoculum producing a detected quantity of $C^{14}O_2$ under controlled conditions. It is conceivable that the radioisotope method could then be used as a standard for the other methods.

While not normally a source of error, another characteristic of the radioisotope coliform test somewhat reduces its sensitivity and creates a minor annoyance. This is true not only of the rapid coliform test, but of any technique using labeled organic compounds. Beta disintegrations impart sufficient energy to adjacent molecules or ions to break bonds. Fragments are thus produced, generally free radicals, which enter into one or a series of reactions, some of which terminate in the production of $C^{14}O_2$. The non-metabolic $C^{14}O_2$ in the medium can be reduced by promoting exchange with the atmosphere through shaking or bubbling with carrier gas. Sterile controls are routinely run with tests for the purpose of determining the levels of nonmetabolic $C^{14}O_2$.

b. Isotope Hazards. A word is in order concerning the hazards associated with handling radioisotopes in the test. The levels of activity used are so small, several microcuries per culture planchet, that a laboratory can conduct experiments with the method without requiring sufficient C^{14} to be kept on hand to warrant a permit from the Atomic Energy Commission although the latter is readily obtainable. Other than normal, sensible care, no special precautions are required with the method. The beta particles emitted by C^{14} are of relatively low energy and are completely attenuated by the flask and planchets containing the radioactive medium. Even in open flasks or planchets, the C^{14} cannot project beta particles beyond several centimeters in air.

The gas produced by the test has a C^{14} content in the order of micromicrocuries. Small amounts of gas which may escape collection are vastly diluted with air. Probably the principal concern associated with the use of isotopes is the realization that "aseptic" techniques must be used out of consideration for isotopic contamination of the test as much as out of consideration for bacteriological contamination. Nonetheless, in keeping with the general philosophy of isotope handling, routine mop-up counting to check against accidental spills is recommended, as is the use of a hood to carry off the minute traces of $C^{14}O_2$.

Upon completion of the test, a drop of disinfectant is added to each planchet to prevent further generation of $C^{14}O_2$. Although the planchet contents could readily be washed down the sink in accordance with AEC standards, the practice followed has been to store all spent radioactive materials and containers for shipment to a commercial isotope disposal center.

c. Radioactive Test Cost. Another factor generally associated with the use of isotopes is high cost. This was true in the early days of the rapid coliform test. The isotope for a single test cost $300.00. The changes reported in the use of the labeled compounds and volumes required have reduced this cost to approximately 10 cents per test. Materials and labor for the test are now less expensive than those for the standard methods.

2. *Total Bacteria Test*

In addition to the needs for the rapid determination of particular species or groups of bacteria, there is also a need for the rapid detection of total bacteria present in a given sample. Classic techniques for total bacteria tests are used in the food processing and serving industries, in testing water supplies and in other public health applications.

Of the six principal elements comprising life (carbon, oxygen, hydrogen, nitrogen, phosphorus, and sulfur) carbon, hydrogen, phosphorus, and sulfur occur in unstable forms. The short half-lives of the phosphorus radioisotopes make their use difficult for routine tests. Furthermore, phosphorus is not evolved in a metabolic gas. S^{35} has a half-life of 87 days which, far short of the convenient 5,568-year half-life of C^{14}, nonetheless, permits its practical use. Obviously, corrections must be applied for the age of compounds containing S^{35}, but the compounds are useful for several half-lives. S^{35}, like carbon, is a beta emitter. Although protein has an average carbon to sulfur atomic ratio of approximately 150, sulfur has 9,300 times the specific activity of carbon. On this basis, sulfur would possess a theoretical advantage of 62 to 1 over carbon for use as a label in living material. In actual practice, carrier-free compounds are never used so that the theoretical specific activity comparisons do not come into direct play although they indicate the, relative specific activities that may actually be attained in carbon and sulfur compounds. Like carbon, sulfur, in appropriate compounds, can be offered to living organisms and subsequently detected in the organisms themselves or in metabolic products. When S^{35} is assimilated by organisms producing H_2S^{35}, the evolved gas can be trapped and counted in the same manner as $C^{14}O_2$ using either barium hydroxide or lead acetate to fix the sulfide.

The radioactive isotope of hydrogen, tritium, offers greater problems to radiomicrobiology, despite its satisfactory half-life of 12.5 years. On disintegration, H^3 yields a very low average energy, 0.018 m.e.v., making counting difficult compared to carbon and sulfur which yield betas with average energies of 0.155 and 0.167 m.e.v. respectively. Tritium exchanges readily, resulting in a loss of the tag from the desired sites and a corresponding contamination of sites where the deposition of radioactive atoms may interfere with the test. However, it has become relatively simple to tritiate organic compounds by virtue of this otherwise undesirable tendency to exchange. Tritium counting has been made easier by commercially available instruments, but the complexity and expense of these instruments still substantially exceed those suitable for carbon and sulfur counting. Nonetheless, the specific activity of H^3 is 2000 times that of C^{14}. It is the most abundant species of atoms present in living material and exceeds carbon 12:7. On this basis, it possesses a theoretical advantage over C^{14} of approximately 3400. This figure and the corresponding one previously cited for H^3 pertain to the apparent advantages. In the case of radioactive gas detection, they would have to be further modified by the fraction of the labeled compound converted to gas. Each application must be considered on its own grounds. Thus, there will be circumstances which will warrant the use of tritium labeling in rapid microbiological determinations. Labeled tritium in appropriate compounds assimilated by cells might conveniently be recovered in gaseous form as $H_2{}^3$, $H_2{}^3S$, $CH_4{}^3$, or $NH_3{}^3$. Appropriate getters would be required. The use of one, two, or all three of these radioactive isotopes in a nonselective medium can produce a total bacteria test. However, unless multiple dilution techniques are used, it is difficult to conceive of a means for making such a test quantitative. The vastly differing metabolic rates and lag periods of diverse species otherwise make it impossible to relate the radioactivity detected to the numbers of cells in the inoculum.

3. *Bacteriological Warfare Defense*

Shortly after publication on the rapid coliform research (Levin *et al.* 1956, 1957) the U.S. Army Biological Laboratories became interested in the possibilities of radioisotopic bacteriology. At this time, the presumptive coliform test was being developed in the apparatus shown in Fig. 4. After inoculation of the culture planchet, the apparatus was assembled. Air was introduced through the side arm, sweeping across the surface of the medium and entraining any gas produced. The air containing the radioactive gas was then exhausted through a barium hydroxide-impregnated, porous paper, collecting pad secured in the top of the device. Using. apparatus patterned after this, Yee *et al.* (1958) at the Army Biological Laboratories tested the method by incorporating cysteine-S^{35} into a medium developed at their laboratory to induce H_2S production by many species of organisms. The paper pad was impregnated with lead acetate. Using *Serratia marcescens* as a test organism, the results shown in Fig. 5 were obtained. Although the labeled cysteine was more than 6 months old, and therefore considerably reduced in activity, as few as 10,000 cells were detected in less than 3 minutes. It is of interest to note that this early H_2S^{35} production abated after 2 or 3 minutes. A similar early "burst" of CO_2 production has been noted with coliform organisms, but since the test for the latter is designed to detect very small numbers of cells, the gas is collected for 4 hours to take advantage of growth. Also worthy of note are the low sterile controls achieved with cysteine-S^{35}.

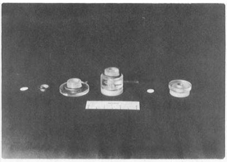


FIG. 4. Apparatus developed for radioisotope test during aeration. The apparatus used by the U.S. Army Biological Laboratories in its radioisotopic total bacteria test was patterned after this. Left to right: filter membrane, culture planchet, base, chimney, porous pad, top. Unit assembles in same order.

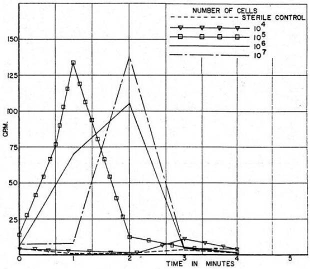


FIG. 5. Response of S. *marcescens* to rapid test using cysteine-S^{35}. Plot was made from data obtained by Yee *et al.* (1958). Courtesy U.S. Army Biological Laboratories.

4. *Exobiology*

Turning from the grim prospects of biological warfare, there is a happier use for a total microbes test. With amazing rapidity, the grandest era of adventure in the history of mankind has dawned. The earliest accounts of man show his yearning for knowledge of the celestial bodies. Within the past few years, fantasy on this subject has been reduced to reality. At this writing, two instrumented vehicles are spanning interplanetary voids, one bound for Mars and the other for Venus. These vehicles, laden with scientific instruments, have been set on courses that will take them close enough to their destinations to obtain reliable scientific data on surface conditions and transmit them back to earth. These "fly-by" vehicles will soon be followed by other space craft which will land instruments on the surfaces of the planets. The question of paramount interest and importance is "Does life exist beyond our planet?" Tentatively designated by the (U.S.) National Aeronautics and Space Administration for the first Mars landing is "Gulliver" (Levin *et al.*, 1962; Levin and Carriker, 1962), a life detection experiment based upon the rapid radioisotope test.

a. Gulliver. An instrument designed to detect life must be based on certain assumed characteristics of that life. Although our imagination can conjure up various exotic forms and mechanisms which would fit our definition of "living," we cannot ignore the rather amazing fact that all the diverse forms of life on earth share common metabolic processes at the cellular level. Despite the manifold possibilities afforded by the range of chemical, physical, climatological, and other environmental conditions on earth, only aqueous and carbonaceous life exists and, to our knowledge, ever existed. No element approaches carbon in its ability to form complex chains, offering almost an infinite variety of macromolecules from which evolution could choose those best serving it. Similarly, water has no peer as a universal vehicle for solutes and, thus, is best qualified to serve the life processes. It is only logical, then, that our first extraterrestrial life explorations be directed toward types of life resembling those we know.

Logic also dictates that extraterrestrial biological forms should be sought at the micro level. These are more likely to be ubiquitous than would macro forms, and would therefore be easier to obtain by the limited sampling techniques permitted by remote or automatic operations. Any biosphere at or approaching equilibrium and containing macro forms would require a device, such as microorganisms, to perform the catabolic processes. Otherwise, the system would be unidirectional, going in the unlikely direction of decreased entropy. The odds against such a short-lived system being in operation at the time of a landing would be great.

Having thus determined to seek microbiological forms possessing a biochemistry similar to or approximating our own, other factors influencing the experiment must be considered. The most likely candidate for extraterrestrial life is Mars. To get there with propulsion equipment now available or under development will require approximately 8 months. It is planned that the space vehicle will fly by or to Mars, and, enroute, dispatch an instrument capsule to land on the planet. Impact will be reduced by the opening of a parachute when the capsule enters the Martian atmosphere. The various instruments contained in the capsule will perform their experiments on battery power and transmit the data to earth by radio. Because of the great thrust required for the journey, most of the space craft will consist of fuel, limiting the capsule to approximately 100 to 300 pounds, including instruments. This imposes severe limitations on the size and weight of the instruments, the amount of power they may draw and the length of time power will be available from the batteries which also suffer from the weight limitation.

Added to these stringent conditions imposed upon a life-seeking experiment are those of shock and vibration experienced upon launch and impact, the hard vacuum of space, and the wide temperature range that must be withstood during flight and on the target planet. Simplicity and reliability must be such that the results can be unambiguously interpreted. The type of signal produced must be simple enough to be accommodated by the capability of the telemetry which will be considerably restricted by the power limitation. To preclude contaminating the planet or spoiling the experiment, it is imperative that the entire capsule and contents be completely free of earth organisms. The instrument and reagents, therefore, must be capable of withstanding severe heat sterilization.

While experiments by Hawrylewicz *et al.* (1962) have indicated that it is possible for earth organisms to grow under Martian conditions, it seems likely that the stringency of the Martian environment would result in fewer organisms per unit surface than on earth. Because of the cold climate, the Q_{10} law would anticipate a lower average rate of metabolism. The weight and power limitations and the rotation of Mars, which will interrupt radio communication when the instrument is on the far side of the planet, indicate that the duration of the experiment may be as little as 4 hours and probably no more than 24 hours. These considerations require that the life detection experiment be sensitive to small numbers of cells and that it have a rapid response time. The radioisotope technique is capable of meeting all of the above criteria.

As opposed to the desired specificity of the rapid coliform test, an early extraterrestrial life detection experiment should support and detect the growth of all types of microorganisms. Thus, it should be even more general than the total bacteria test. Gases are common products of metabolism of microorganisms. Moreover, most species, and possibly all, produce carbon dioxide. This includes photosynthetic organisms. Other gases of metabolic origin which can be readily labeled are methane, ammonia, hydrogen sulfide, and molecular hydrogen. Research efforts have been directed toward developing a broadly nonselective microbiological medium. The inclusion of appropriately labeled compounds results in the production of labeled gas.

Radioactive substrates tested in complex media have included sodium formate-C^{14}, uniformly labeled glucose-C^{14}, sodium acetate-1-C^{14}, sodium-pyruvate-1-C^{14}, glycine-2-C^{14},. cysteine-S^{35}, a yeast extract randomly labeled with C^{14}, and an *E. coli* extract randomly labeled with C^{14}. A combination of formate-C^{14} and uniformly labeled glucose-C^{14} has produced the best results in the medium shown in Table IV. With this medium, rapid responses have been obtained from a wide range of representative microorganisms including bacteria and other fungi, streptomycetes, and algae. Species successfully detected include aerobes, anaerobes, facultative anaerobes, thermophiles, mesophiles, psychrophiles, heterotrophs, phototrophs, autotrophs, spore formers, and nonspore formers. Table V presents results obtained from a wide range of test organisms. Response times and activity levels are shown to illustrate the type of data obtained by the tests. No attempt was made to relate the response to the size of the inoculum. These responses have been obtained by the detection of $C^{14}O_2$ only. This does not, however, preclude the possibility of including additional labeled compounds to produce other radioactive gases. Doing so would improve the probability of response from unknown organisms and also increase the sensitivity. Because of their metabolic importance, methane and hydrogen sulfide are the two most likely candidates in this regard. Figure 6 is a photograph of the instrument[1] being developed for the experiment. An exploded view showing the parts and assembly, together with a schematic diagram, is shown in Fig. 7. The instrument weighs approximately 1½ pounds. Two units will be contained in the space capsule, one to serve as a test and the other as a control. The experiment will proceed as follows:

TABLE IV
RADIOACTIVE TEST MEDIUM USED IN "GULLIVER"

Component	Amount
K_2HPO_4	1.0 gm.
KNO_3	0.5 gm.
$MgSO_4.7H_20$	0.2 gm.
NaC1	0.1 gm.
$FeCl_3$	0.01 gm.
Amino acid hydrolyzate	4.0 gm.
Yeast extract	13.0 gm.
Soil extract	250.0 ml.
Proteose peptone No. 3	20.0 gm.
Malt extract	3.0 gm.
Ascorbic acid	0.2 gm.
L-Cystine	0.7 gm.
Beef extract	3.0 gm.
Glucose-C^{14}	0.05 gm.
Na Formate-C^{14}	0.02 gm.
Distilled H_2O	up to 1 liter
Total activity	10 μc/ml.

TABLE V[a]
ORGANISMS EVOLVING $C^{14}O_2$ WHEN TESTED IN THE MEDIUM OF TABLE IV

Organism	Activity above that of control (counts per minute)	
		Response within 3½ hours
Arthrobacter simplex	1,629	
Azotobacter agilis	28,956	
Azotobacter indicus	1,868	
Bacillus subtilis spores	11,784	
Bacterium bibulum	7,221	
Chlorella sp.	323	
Clostridium pasteurianurn	1,698	
Clostridium roseum	5,367	
Clostridium sporogenes	664	
Escherichia coil	65,389	
Micrococcus cinnabareus	479	
Mycobacterium phlei	1,913	
Pseudomonas delphinii	971	
Pseudomonas fluorescens	6,701	
Pseudomonns maculicola	16,266	
Rhodopseudomonas capsulata	365	
Rhodospirillum rubrum	420	
Saccharomyces cerevisiae	858	
Staphylococcus epidermidis	3,219	
Streptomyces fradiae	560	
Xanthomonas beticola	58,189	
Xanthomonas campestris	537	
		Response within 6 hours
Photobacterium phosphoreum	2,423	
Thiobacillus novellus	141	
Thiobacillus thiooxidans	102	
		Response between 6 and 24 hours
Rhizobium leguminosarium	1,123	

[a] From Levin *et al.* Reproduced courtesy of *Science* **138**, 3537, 117 (1962).

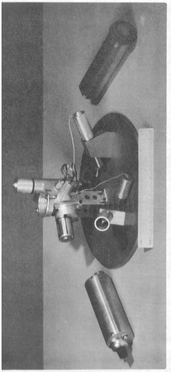
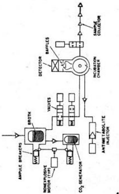


FIG. 6. Gulliver. A view of the complete instrument and associated electronic systems. Projectile guns would be mounted adjacent to capsule wall. Base plate and projectile gun mounts are for display only. Total flight weight is approximately 1.5 pounds. Instrumentation by American Machine and Foundry Company, Alexandria, Virginia, Division.



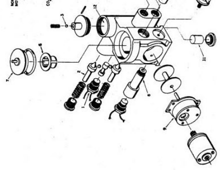


FIG. 7. An "exploded" diagram of the instrument now being used. 1, Nonexplosive motor; 2, ampule breakers; 3, pressure release valve; 4, normally closed valve; 5, normally open valve; 6, throat baffle; 7, radiation detector; 8, antimetabolite injector; 9, electric motor and gear box; 10, thermostat; 11, acid ampule; 12, broth ampule; 13, string port. From Levin *et al.* (1962), p. 118.

When the capsule containing Gulliver comes to rest on the surface of Mars, a glass ampule containing sterile radioactive broth is broken. Carrier gas is bubbled through the broth to remove traces of nonmetabolic radioactive gas which may have formed due to some breakdown of the medium by internal and external sources of radiation during the long voyage. While the broth is purged, the two projectiles are fired. Each extends a 25-foot-long string over the surface of the planet. The strings are coated with silicone grease so that particles contacted are retained. A tiny motor then winds the strings into the incubation chamber together with their precious cargo of soil. This operation takes several minutes, during which the culture chamber is free to exchange its atmosphere with that of Mars. During the test, the only condition which will be imposed on the ambient environment of Mars will be the maintenance of the broth above the freezing temperature. After the string has entered the chamber, the chamber is sealed and a background count of the radioactivity is made. The radioactive broth is then injected into the culture chamber, saturating the string. A radiation detector is mounted directly above the culture chamber. A baffle intervenes so that the detector cannot see the radioactivity in the broth. The face of the detector is thinly coated with barium hydroxide.

If organisms which can utilize the medium are present on the soil particles, $C^{14}O_2$ should be evolved. The gas will migrate from the culture chamber through the baffle arrangement and deposit on the coating of barium hydroxide where it will be fixed as carbonate. The radiation detection instrument will make periodic counts of the amount of $C^{14}O_2$ thus deposited. Should other gases be sought through the use of additional labels, appropriate getters will be applied to the face of the radiation detector.

The second instrument will be identical to the first and will be operated in the same manner with the exception that an antimetabolite will be injected shortly after introduction of the Martian soil. The data from each will be transmitted to earth. The generation of the classic biological growth curve when radioactivity is plotted as a function of time will constitute evidence of life in the test instrument. If an inhibition in the growth curve is produced in the control, a very strong case will have been made for life on Mars.

The instrument shown in Fig. 6, containing an ampule of the medium cited in Table IV, has been field tested. The results of such a test, performed on frozen soil, are shown in Fig. 8. After collection outdoors, the instrument was removed to the laboratory where the experiment continued at room temperature. The curve produced is interesting from several aspects. The rapid response is evident. Moreover, three exponential phases of the curve are evident on the semilog plot. The indication is that three different groups of organisms predominated in the sample, each having its distinct lag period, exponential growth phase, and stationary phase. In addition, the average generation period for each group of organisms is given by the slope of the respective portion of the curve. Furthermore, it is interesting to note that the generation periods for the exponential phases increased from left to right on the time scale. This is as might be expected since it associates those organisms which took longer to come out of lag phase with slower rates of metabolism.

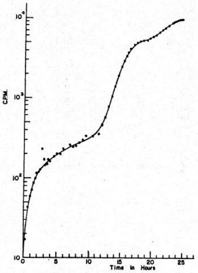


FIG. 8. Evolution of $C^{14}O_2$ by microorganisms in soil collected and cultured in a field test with the instrument shown in Fig. 6. The soil was collected from frozen ground. From Levin *et al.* (1962), p. 119.

Rapid responses have been obtained with the method when strings were drawn across various adverse environments such as a pile of sand, an asphalt street, and a plate glass window. As little as 10 mg. of soil supplied from a remote area of the Mojave Desert and aseptically stored for several months produced the response seen in Fig. 9. In this case, the labeled compounds in Table IV were increased fivefold in concentration. Despite the gratifying rapidity of the various results shown, Gulliver is still one to two orders of magnitude less sensitive than the planchet method. This is due to factors introduced by the geometry and components necessary to the function of the instrument. Attempts are underway to increase the sensitivity of the instrument until it closely approaches that offered by the basic technique. Similarly, considerable effort will yet be spent in improving the medium to increase sensitivity and the broadness of response to it. When the final medium has been developed, those labeled and unlabeled compounds which have optical activity will be racemized in the event that Martian organisms require isomers opposite to those utilized on earth.

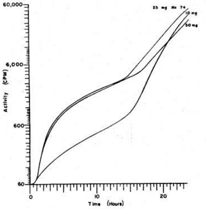


FIG. 9. Evolution of $C^{14}O_2$ by organisms in various quantities of Mojave Desert soil inoculated into medium shown in Table IV with formate-C^{14} and glucose-C^{14} concentration increased fivefold. Curves are drawn through points obtained from readings taken every 24 minutes. From Levin *et al.* (1962), p. 116.

Should Gulliver find life on Mars, the door would be open to extensive microbiological determinations on Mars. Rather than an "organic smorgasbord," specific labeled substrates would be offered to the organisms. Temperature and light responses would be measured. The organisms would be tested under aerobic and anaerobic conditions and under atmospheres of various compositions. Specificity for optical isomers would be determined, DNA-like compounds would be sought. These and other experiments might determine whether the Martian organisms shared their origin with life on the earth, or whether life evolved independently on the two planets. Such genetic relationship or independence would be of major importance in the search for the origin of life.

5. *Selection of Antibiotics*

Another application of the radioisotope technique may provide clinical medicine with an important tool. Just as the introduction of a tag can quickly detect growth in microorganisms, the inhibition of growth can be detected with equal ease. A method has been developed by Heim *et al.* (1960) which demonstrates this. The apparatus is the same as that described for the rapid coliform test. However, filtering through a membrane filter is not required since the numbers of organisms available are large.

Two-tenth ml. replicates of organisms isolated from the infected person are placed into a set of the one-inch planchets, each of which contains 0.8 ml. of trypticase-soy broth to which has been added 0.003% sodium formate-C^{14} (2 mc./millimole). Two or more replicates are used for the inoculated control. Into a series of sets of replicates of the culture, various concentrations of the antibiotics to be tested are added. Replicate sterile controls are also run. All planchets are then incubated at 37°C. in petri dishes. At the end of 2 hours, planchets containing pads impregnated with a saturated solution of barium hydroxide are inverted over the incubating planchets. Collection of $C^{14}O_2$ thus proceeds for 30 minutes after which the collection planchets are removed, dried, and counted for radioactivity. Incubation is continued and a similar collection of $C^{14}O_2$ is made at the fourth hour, terminating the test.

The effects of various concentrations of penicillin and tetracycline on an *E. coli* are shown in Table VI. The table includes the results obtained when replicate concentrations of the organisms and antibiotics were tested by the conventional 24-hour tube dilution technique. An inoculated control, to which no antibiotics had been administered serves as a reference. All values reported for the radioactive test are averages of duplicates from which sterile control and background levels have been subtracted. A decrease in fourth hour activity over that of the second hour indicates effectiveness of the antibiotic as applied. The difference between penicillin and tetracycline is thus readily apparent. The increase in activity of the portions containing the lesser quantifies of penicillin over those containing no antibiotic has been frequently observed. This is an interesting revelation of the radioisotope technique and requires further study for interpretation. The results of the 24-hour tube dilution test were in complete agreement with the rapid test and verified that tetracycline became effective at a concentration of 5 µg. per ml. Table VII shows the results obtained by the test performed on *Proteus*. Both the *E. coli* and the *Proteus* were isolated from hospital patients. Other tests have been successfully performed directly on body fluids, including urine. Such direct application is the ultimate objective of the rapid method.

TABLE VI[a]

EFFECTS OF ANTIBIOTICS ON $C^{14}O_2$ RELEASED BY *E. coli* IN 2 AND 4 HOURS

		Counts Per Minute[c]		Tube Dilution,
Antibiotic	Concentration[b]	2 hours	4 hours	24 hours
None	—	1915	15,810	—
Penicillin	1 unit/ml.	2654	25,406	Growth
	5 units/ml.	1379	23,105	Growth
	50 units/ml.	4665	9,854	Growth
Tetracycline	1 µg/ml.	273	1,588	Growth
	5 µg/ml.	348	16	No growth
	50 µg/ml.	17	12	No growth

[a] From Heim *et al.* Reproduced courtesy *Antimicrobial Agents Ann.* p. 124 (1960).

[b] Concentration of cells, 10^{-2} dilution of an 18- to 20-hour culture.

[c] Counts per minute have background and sterile controls subtracted and are averages of duplicates.

6. *Prospecting for Petroleum and Gas*

A radioisotope technique for prospecting for petroleum has been developed by Davis (1957). Earth overlying oil or gas deposits is permeated with hydrocarbons emanating from the deposits. The earth in these areas contains hydrocarbon-consuming microorganisms which have been selected by the environment. A sample of earth overlying a suspected petroleum or gas deposit is exposed to radioactive hydrocarbons in gaseous or liquid phase. If such organisms are present, the label will be detected in the metabolic products. The detection of these products indicates the presence of the deposit.

7. *Experimental Uses*

Until now the discussion of application of the radioisotope technique has been limited to its use in practical test methods. However, the sensitivity and simplicity of the method make it valuable in fundamental as well as applied research in microbiology. It can be useful in conjunction with conventional respirometric methods. It also offers some advantage over these. For example, it permits results to be obtained within minutes, or even seconds, of the onset of an experiment. This makes possible a study of the early kinetics of metabolic reactions otherwise difficult or impossible to observe. An example of this is the early "burst" of activity detected when coliform organisms or *S. marcescens* (as reviewed herein) are given a source of energy. It is possible to study minute details of respiration rate changes in the organisms when making the transition from lag to growth phase. Automatic instrumentation such as that shown in Fig. 10, developed especially for laboratory research on the Mars life probe, makes it possible to follow gas production as it occurs. This permits experiments to be conducted on organisms at specified points in the development of the culture. It is possible to time and measure the effects produced by the introduction of various metabolites or antimetabolites of interest.

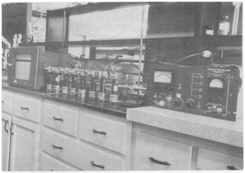


FIG. 10. Automatic radioisotope microbiological monitoring apparatus. Multichannel recorder is at left. Eight culture chambers, same size and design as in Gulliver, are shown in center. A geiger tube (black cylinder) is mounted directly over each chamber. Log count rate meter is at right. Gas produced by cultures in each of the chambers is sequentially counted and recorded. Each chamber is counted at 24 minute intervals.

An interesting effect observed by the radioisotope technique was that CO_2 is required by growing coliform organisms. When the planchet apparatus was first used, the barium hydroxide planchet was inverted over the culture planchet immediately after inoculation. This seemed logical to effect maximum $C^{14}O_2$ collection. However, cultures containing small numbers of cells failed to go into exponential growth under these conditions. This inhibition was removed when the culture was permitted to incubate before application of the CO_2 collection planchet. Further evidence of this effect was produced by comparison of $C^{14}O_2$ production by sterile controls and test cultures several minutes after inoculation. It was found that less $C^{14}O_2$ was collected from the cultures than from the sterile controls. The nonmetabolic $C^{14}O_2$ in the sterile controls had been incorporated by the cells. This is in keeping with recent findings cited by Roberts *et al.* (1955, p. 95) of the importance of CO_2 in anabolism.

Another type of experimental application is the study of toxicity resulting from the membrane filter. Since the membrane filter is an integral part of the rapid coliform test, it is important that the toxic effect be reduced to a minimum. At the same time, insight thus gained will help elucidate the results obtained with the standard method membrane filter test.

III. Conclusion

It is abundantly clear that there is great room and need for improvement in classic procedures for quantitative microbiological determinations. Radioisotope techniques which, through their extreme sensitivity, render extended incubation of organisms unnecessary, may meet the important requirement for speed. Accuracy, another major requirement, may also be significantly improved by using isotopes to investigate "biological vagaries" and errors introduced through imperfect methodology. Finally, continued exploitation of isotopes in microbiological research promises to elucidate many fundamental biochemical processes.

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[1] Instrumentation is being performed by American Machine and Foundry Company, Alexandria, Virginia.