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A Radioisotope Technic for the Rapid Detection of Coliform Organisms

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The authors point out that their method for the rapid detection of coliforms in drinking water may have other applications in food and milk quality control. If the method should work for total bacteria determinations, it would have important civil defense implications.

The fact that it is impossible to determine the bacterial quality of drinking water at the time it is consumed is a cause of constant and major public health concern. With the increase in size and complexity of municipal water supply systems the public health hazards and consequences of contamination have become ever greater. Should contamination be accidently or deliberately introduced into a water system, routine bacteriological examination would not detect the dangerous condition until two or three days later. By that time the water might have been distributed and consumed over a large portion of the community. For this reason much attention has been focused on the need for reducing the length of time required for bacteriological analyses. The method described in this paper is an attempt to shorten the presumptive test for coliform organisms to one hour or less.

The delay in obtaining results by all conventional methods is due to a common cause, namely, that the criteria for the determination require direct visual evidence of colonies or gas production by the bacteria. Quantitative determination by the standard method test [1] requires that a single coliform bacterium must give rise to a population of 1,700,000,000 cells [2] before the accumulation of evolved gas is sufficient to produce a visible bubble. Similarly, the membrane filter test [1] requires an incubation period of 20 hours to allow the development of visible colonies. Almost all the time required for either of these tests is spent in waiting for the tremendous amount of bacterial reproduction which must occur to produce the necessary visual evidence.

It is unlikely that any great increase in bacterial generation rates can be induced. Any significant reduction in time required for bacteriological analysis, therefore, must be achieved through the development of more sensitive means for detecting bacterial metabolism. The authors have been working toward this goal through the use of radioisotope technics.[3, 4]

Radioisotope detection instruments are capable of sensing particles many trillions of times smaller than a bacterium. If bacteria would incorporate unstable atoms, radiation from the harvested cells or from their metabolic products would indicate the presence of the bacteria. Isotopes have been used

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to study metabolic pathways of various compounds and elements assimilated by large numbers of bacteria.[5]

If selected isotopes in quantities sufficient for subsequent detection were assimilated by small numbers of bacteria, a rapid method for bacterial determination might result. For example, if coliform organisms would ferment lactose made radioactive by the substitution of a carbon atom with carbon-14, the carbon dioxide evolved by the cells should be radioactive. Calculations based on reported respiration rates of E. coli [6] indicated that the method was feasible. Since the fermentation of lactose with the production of gas containing carbon dioxide is the standard method [1] presumptive test for coliform organisms, the radioactive technic should therefore fulfill the requirements for that test.

Experimental work was begun designed to test the feasibility of the proposed method. Since the approach represented a departure from orthodox bacteriological procedures, emphasis to date has been on questions of practicality and economy—statistical and quantitative aspects have not been stressed. In the year and a half since laboratory work began considerable changes and refinements have been made in the method.

As last reported,[4] apparatus for the method consisted of two test tubes connected in series with a tube of glass beads and a paper fiber pad holder to form an air train. Air from the laboratory supply was metered by a valve controlling the bubble rate in the first tube. The air was introduced into the second tube through a fritted glass diffuser. This tube contained standard [1] lactose broth made with 1-C^{14} lactose [7] obtained from the National Bureau of Standards. Upon leaving the second tube the air passed through the glass beads (to remove any air-borne lactose droplets) and escaped to the atmosphere by passing through the paper fiber pad. When a test was made a membrane filter, through which water suspected of containing coliform organisms had been passed, was immersed in the tube containing the lactose broth, and the paper pad was impregnated with a saturated solution of barium hydroxide. The tube containing the membrane filter was incubated at 37° C and aeration was maintained. If coliform organisms were present, $C^{14}O_2$ was evolved and carried in the air stream. Upon meeting the barium hydroxide solution, the $C^{14}O_2$ precipitated on the pad as barium carbonate. The pad was replaced at intervals, and the exposed pads dried and counted for radioactivity in a gas-flow Geiger counter. A significant level of radioactivity in the pad constituted a positive presumptive test for coliform organisms.

Results of tests with this apparatus were highly encouraging, frequently producing results within one hour even when the total inoculum was of the order of 100 cells. Several aspects of the test warranted further refinement. Barium hydroxide impregnated pads through which air from sterile controls passed became radioactive, indicating that radioactive gas of nonmetabolic origin was evolved by the lactose itself. It was, therefore, necessary to subtract the radiation level of the sterile control from the radiation level produced by the test. The nonmetabolic evolution of radioactive gas masked the test somewhat by increasing the time necessary for the radioactivity of the test to reach a significant level. Another masking factor was the solubility of carbon dioxide. A substantial amount of the radioactive carbon dioxide evolved by the test organisms was held in solution in the lactose broth, again delaying the point of determination. An additional important consideration was the economic one, in that the 1-C^{14} lactose was quite expensive.

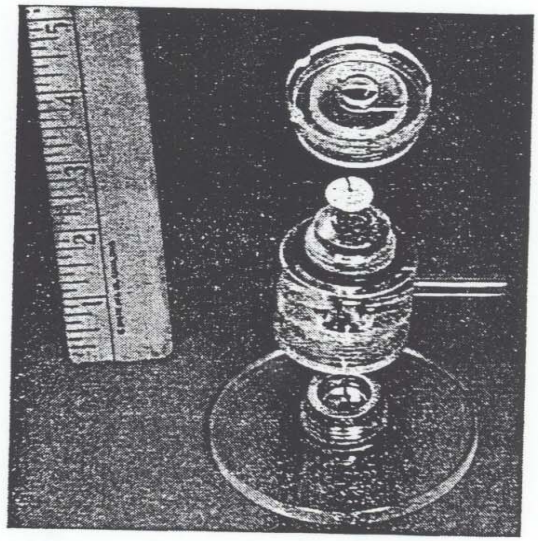
A great deal of work was done in

trying to reduce the nonmetabolic evolution of radioactive gas from the 1-C^{14} lactose. It seemed likely that there were two principal sources of this evolution. One was that oxidation-reduction reactions involving the lactose might have been induced by the presence of various metallic ions in trace amounts. Such ions could have been introduced into the lactose by the walls of the glass vessels in contact with it. The second source was the destruction of chemical bonds of the lactose by radiation from its own disintegration. Such self-induced degradation of radioactive organic compounds has been reported.[8] This type of decay could account for a substantial portion of the nonmetabolic radioactive gas.*

Tests showed that the nonmetabolic evolution of radioactive gas was, as might be expected, roughly proportional to the volume of 1-C^{14} lactose broth producing it. The other problems cited above were also related in direct proportion to the volume of 1-C^{14} lactose broth—the amount of $C^{14}O_2$ retained in solution and the cost of the test. On the other hand, the objective of the test, the production of $C^{14}O_2$ by metabolic activity of coliform organisms, was independent of the volume of the medium, provided the volume was not reduced below the very small amount necessary to sustain the cells. A major reduction of the quantity of radioactive medium used in the test was indicated.

Originally the test had been run in 10 ml portions of 1-C^{14} lactose broth. At the time of the last report [4] the volume had been reduced to 5 ml to conserve the isotope. Now, however, a true semimicro technic was desired. Tests showed that the glass-bead vapor trap was not necessary. Moist paper pads were placed very close to portions of aerating 1-C^{14} lactose broth and no trace of lactose was found on the pad by chromatographic or radioactivity tests. This presented the possibility of consolidating and reducing the size of the apparatus. One difficulty was the impracticality of aerating small quantities of medium with fritted glass diffusers. It was believed that if the depth of 1-C^{14} lactose broth over the organisms was very shallow the $C^{14}O_2$ evolved would diffuse to the surface rapidly. Air blown across the surface of the broth could carry off the $C^{14}O_2$ as it emerged and the concentration gradient of the gas would be maintained in the

Figure 1—Detection Unit



Close-up of base with planchet inserted, middle section showing air-injection tube (fine holes in end of tube direct air downward to surface of radioactive lactose broth in planchet), paper fiber pad, and top section. For test 0.1 ml 1-C^{14} lactose broth is pipetted into planchet; membrane filter containing organisms or inoculum of organisms is placed in the broth; one drop of barium hydroxide solution is added to pad; and unit is assembled. Air hose is connected and unit immersed up to top of base section in 37° C water bath. Vertical wire and hole in planchet were necessary to produce "exploded" view. Scale is in inches.

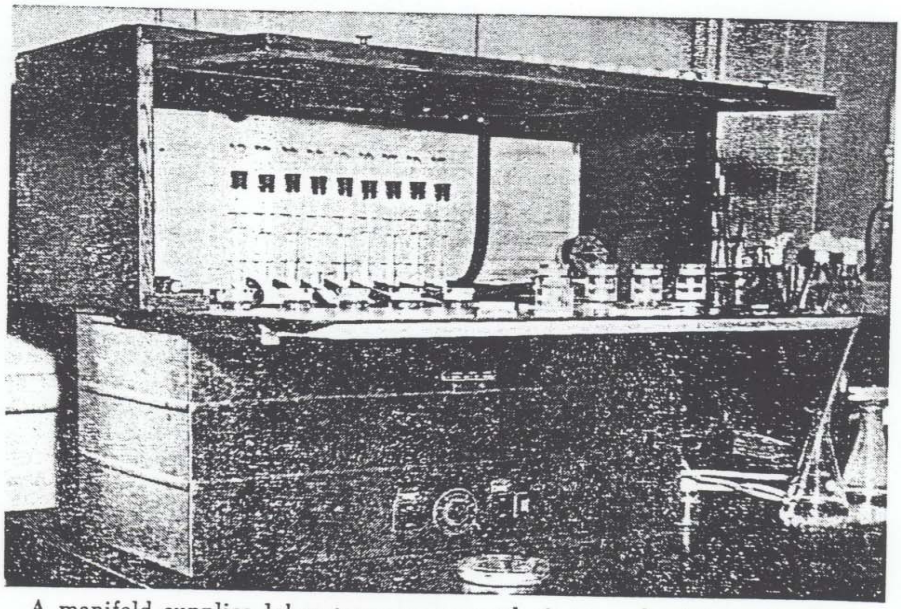
* Personal communication, B. M. Tolbert, Radiation Laboratory, University of California, Berkeley, Calif.

direction that would remove it from solution.

The apparatus developed was similar to, but larger than, that shown in Figure 1. The 1-C^{14} lactose broth was placed in the bottom section in a small cup, or planchet, of the same type used in the counting apparatus.[4] The bacteria were inoculated into the broth by the addition of 0.1 ml of a culture suspension or, in the case of a water sample test, by the immersion of a membrane filter through which the suspected sample had been filtered. Air, introduced through the middle section, was blown downward over the surface of the broth when the sections were fitted together. The paper fiber pad, impregnated with saturated barium hydroxide solution, was held in the top section. By removing the top section the pad could be changed at desired intervals, dried for 10 minutes, and counted for radioactivity. Five-minute counts were sufficient for the accuracy required. When tests were being made the unit was immersed in a water bath incubator to the depth of the bottom section. The apparatus reduced the amount of 1-C^{14} lactose used per test to 0.8 ml.

Some extremely interesting test data were obtained. The literature [6] had indicated that the respiration rates of E. coli were approximately the same, whether the organisms were in the lag phase or in exponential growth. In view of this no attempt had been made previously to control the growth phase of the test organisms, though with few exceptions, the cells had been in exponential growth at the time of inoculation. This was because the organisms were grown in nutrient culture until desired concentrations were attained as indicated by optical density tests. When this occurred the organisms were in ex-

Figure 2—Battery of Detection Units (Five in Water Bath, Four on Operating Shelf.)



A manifold supplies laboratory compressed air to each of the units through individual needle valves and bubble meters (the vertical test tubes). A blower-exhausted hood (door shown open) made from a bookcase section encloses the surface of the water bath incubator.

ponential growth. Portions of the culture were quickly diluted to obtain the cell population range selected for the tests. When placed in the broth the organisms continued growing exponentially as indicated by the logarithmic increase in the amount of radioactive carbon dioxide produced with time. Since it is believed that the radioactive method can provide more exact respiratory data than classical methods, particularly in the largely unexplored early minutes of incubation, tests were made of small numbers of organisms inoculated in the lag phase. Organisms present in a sample of drinking water or raw water are likely to be in lag phase and thus the tests would more closely approximate actual conditions under which the method would be used.

In one instance a dilution of E. coli cells was made from an 18-hour nutrient broth culture. The suspension was chilled for three hours at 5° C to induce lag. Similarly chilled sterile tap water was then used to make 10^8, 10^9, and 10^{10} serial dilutions of the suspension. Cell populations of the dilutions were determined by plate counts, the results of which were not known until 24 hours after the test. Into each of four planchets was pipetted 0.8 ml of sterile 1-C^{14} lactose broth. The lactose broth contained 0.3 per cent of 1-C^{14} lactose, the specific activity of which was 3.01 microcuries per milligram. Thus, 7.2 microcuries were contained in each planchet. To each of three of the planchets 0.1 ml of the chilled bacterial suspension was added. The fourth received 0.1 ml of sterile tap water and was maintained as the control. When the inoculations were made the units containing the planchets were simultaneously incubated at 37° C under constant aeration at a rate of three bubbles per second. The barium hydroxide treated pads were replaced every 10 minutes. Table 1 shows the results obtained. In 10 minutes the amount of $C^{14}O_2$ collected from each of the cultures was sufficient to determine that the presumptive test for coliform organisms was positive. The plate counts subsequently revealed that the numbers of organisms present in the test portions

Table 1—Presumptive Test for E. coli in Lag Phase

	Radioactivity *—(cpm †)							
	Approximate No. of Cells							
	115		40		20		Control	
Time (Minutes)	Increment	Cumulative	Increment	Cumulative	Increment	Cumulative	Increment	Cumulative
10	65	65 ‡	83	83 ‡	72	72 ‡	17	17
20	35	100	33	116	37	109	12	29
30	32	132	36	152	26	135	15	44
40	34	166	34	186	41	176	11	55
50	25	191	19	205	17	193	7	62
60	29	220	31	236	30	223	8	70

* Radioactivity is measured above a background of 20 counts per minute.
† The abbreviation cpm stands for counts per minute.
‡ Point of presumptive test determination.

of the 10^8, 10^9, and 10^{10} dilutions were of the order of 115, 40, and 20 cells, respectively. A duplicate run, made with approximately 20, 15, and five cells in lag phase, also produced 10-minute determinations in each of the three populations as shown in Table 2.

Other runs, however, have taken as long as four or five hours to produce positive results. Contrary to what was first expected the time required for positive results was consistantly longer when the E. coli were in exponential growth than when they were in lag phase. An example of such a run which also was one that produced comparatively late results is shown in Table 3. In this case an exponentially growing culture of E. coli in lactose broth was serially diluted 10^5, 10^6, and 10^7 times. Lactose broth was also used as the diluent in order to maintain the cells in logarithmic reproduction. The suspensions were not chilled but, immediately upon preparation, 10 ml of each were filtered through a membrane filter. Each filter membrane was then immersed in a planchet containing 0.9 ml of the 1-C^{14} lactose broth. A fourth 0.9 ml portion of the broth was maintained as a sterile control. Plate count determinations subsequently revealed that the 10^5, 10^6, and 10^7 dilutions contained approximately 13,000, 4,800, and 1,680 cells for the respective 10 ml filtered portions. The test was considered positive for the 13,000-cell culture in 30 minutes, for the 4,860-cell culture in 120 minutes, and not until 240 minutes for the 1,680-cell culture.

Several observations are worthy of note. The high rates of $C^{14}O_2$ production seem to occur initially and then fall off in the lag phase organisms. The evolution of $C^{14}O_2$, as measured by counts per minute evolved per cell per hour, is much higher for lag cells than for exponentially growing cells. This measurement is also considerably higher for cells in low concentrations than for those in cultures where the total populations are higher. In some runs there have been periods during which the $C^{14}O_2$ production of the test samples dipped significantly below that of the sterile control. One hypothesis that can

Table 2—Presumptive Test for E. coli in Lag Phase

	Radioactivity *—(cpm †)							
	Approximate No. of Cells							
	20		15		5		Control	
Time (Minutes)	Increment	Cumulative	Increment	Cumulative	Increment	Cumulative	Increment	Cumulative
10	73	73 ‡	18	18 ‡	39	39 ‡	1	1
20	20	93	20	38	80	119	0	1
30	0	93	9	47	1	120	0	1
40	2	95	6	53	0	120	0	1
50	0	95	1	54	0	120	0	1
60	3	98	22	76	21	141	0	1

* Radioactivity is measured above backgrounds ranging from 19 to 28 cpm.
† The abbreviation cpm stands for counts per minute.
‡ Point of presumptive test determination.

relate these seemingly anomalous findings is that reproducing cells and cells preparing to reproduce incorporate or conserve large quantities of CO_2 for use in building cellular material. Lag phase cells might not exert such a CO_2 demand.

Minute gas leaks which developed in the handmade apparatus frequently marred test runs by allowing $C^{14}O_2$ to escape without passing through the paper fiber pad. Also, tests showed that there was room for further reduction in the quantity of 1-C^{14} lactose required per test. The latter would reduce the cost and at the same time increase the sensitivity by lowering the rate of nonmetabolic evolution. The development of smaller, gas-tight apparatus was needed. In addition, the possibility of utilizing the Robinson gas-flow counter [9] seemed promising. By virtue of its small counting chamber volume the background of the counter averaged approximately five to six counts per minute compared to the 20 to 25 counts per minute background of the counting apparatus then in use. This considerable reduction in background would allow low-level test emissions to become significant earlier since significance is based on the ratio of the test to the control level. A modified Robinson gas-flow counter manufactured at the National Institutes of Health was made available by the Public Health Service. It is operated in the proportional region and is used with a commercially available scaler equipped with an amplifier.

The radioactivity counting equipment is shown in Figure 3. The apparatus shown in Figure 1 was designed around the half-inch planchet used by the Robinson counter. The same type of planchet is used to hold the bacterial cultures as is used to place the radioactive paper pads in the counting chamber. Machined of plexiglass the sections of the unit fit together with screw threads and rubber o-ring gaskets. Paper fiber pads and membrane filters

Table 3—Presumptive Test for E. coli in Exponential Growth

	Radioactivity *—(cpm †)							
	Approximate No. of Cells							
	13,000		4,800		1,680		Control	
Time (Minutes)	Increment	Cumulative	Increment	Cumulative	Increment	Cumulative	Increment	Cumulative
30	24	24 ‡	12	12	2	2	12	12
60	20	44	11	23	0	2	9	21
90	46	90	17	40	1	3	14	35
120	124	214	46	86 ‡	3	6	17	52
150	377	591	51	137	13	19	26	78
180	577	1,168	51	188	32	51	15	93
210	1,847	3,015	85	273	50	101	24	117
240	2,600	5,615	120	393	102	203 ‡	16	133

* Radioactivity is measured above a background of 23 cpm.
† The abbreviation cpm stands for counts per minute.
‡ Point of presumptive test determination.

were obtained in the half-inch size. The pads are made of the same stock as are nutrient pads used in the membrane filter technic. Syringe adapters are utilized to force several milliliters of bacteria-containing water through the filters. As previously, the filter membranes are then placed in planchets containing 1-C^{14} lactose broth. The planchets are fitted into the bottom sections of the apparatus. Paper fiber pads are placed in the top sections and moistened with one drop of a saturated solution of barium hydroxide. The unit is then assembled and placed in the water bath. There are two tops for each unit so that the whole section can be replaced, reducing the delay formerly experienced in changing the pads.

The new apparatus (Figure 1) requires only 0.1 ml of 1-C^{14} lactose per test. This is a 100-fold reduction over the quantity originally used, and a 50-fold reduction since the last report.[4] The per test cost has been reduced by the same amounts since the chief cost is the isotope used.

At the present time the National Bureau of Standards is the only source of 1-C^{14} lactose which is synthesized on a small batch basis. Total costs in producing the 1-C^{14} lactose are such that the radioactive method can compete favorably on a commercial basis with the standard method test. The amounts and activity levels of the 1-C^{14} lactose used do not require special safety precautions other than the ordinary exercise of care. The hood (Figure 2), while not needed to meet exposure limits,

Figure 3—Radioactivity Counting Equipment

The Modified Robinson gas-flow counter is directly below the electrical outlet. A planchet with exposed pad is shown in base plate of counter prior to being rotated into counting chamber. Binary scaler with built-in amplifier is right of counter. Unit on top of scaler is preset timer. Tank to right of scaler supplies counter with gas. The equipment is operated in the proportional range. Beaker at left of picture contains counted pads and planchets ready for disposal.

was constructed on the assumption that even very small amounts of radiation should be avoided if possible.

Several important problems remain to be solved in the rapid presumptive test for coliform organisms. The reason for the apparent biological vagary of the cells in low concentration must be explored. Otherwise, the fact that occasionally low numbers of organisms require several hours to produce positive results will require that samples yielding negative results be monitored for a like period of time. The difference in rates of CO_2 production and incorporation or retention between lag and growth phase organisms requires investigation. Unless this phenomenon is understood it will be difficult to quantitize the test.

Some poisons, in proper concentration, are known to prevent growth and reproduction of bacteria while allowing respiration to continue for several hours.[10] In effect, then, such poisons should prevent the CO_2 incorporation or retention, since it cannot be used to build new cellular material. The incorporation of a poison of this type into the medium might tend to stabilize the rate of CO_2 production at the high lag phase level. It is possible that the poison would also reduce the variation in the amount of CO_2 produced per cell per hour in high- and low-cell density tests. A high-cell density test might evolve less CO_2 due to the fact that the CO_2 produced may be incorporated by nearby organisms. Blocking of the incorporation could produce the equalization of rates.

The new apparatus has only very recently been obtained, but it has been used for several experiments designed to test this possibility. The poison selected was 2,4 dinitrophenol which was added to the 1-C^{14} lactose broth. Equal inocula were added to culture planchets containing the poisoned broth and to culture planchets containing broth without poison. The few tests made showed considerably higher rates of $C^{14}O_2$ evolved in the poisoned cultures than in the controls.

One factor which complicates quantitizing is the difficulty of knowing the exact number of organisms actually present in a calibration run. Regardless of the method used, tests of aliquots of low-population density inocula do not indicate with sufficient accuracy the numbers of organisms in a test portion. One method considered for solving this problem was to continue to incubate the membrane filters used in the radioactive test until colonies appeared. The gas evolved in the rapid test could be correlated with the number of colonies that subsequently appeared. The assumption would be that the number of colonies was the same as the number of cells originally present. This, too, has limitations in that organisms may live for several minutes or hours evolving detectable amounts of radioactive carbon dioxide and yet die without giving rise to a colony. This would be particularly true if a poison should be incorporated into the 1-C^{14} lactose broth.

Discussion

In its present state of development the radioisotope method can be relied upon for rapid qualitative presumptive determinations of gross coliform contamination. Positive results have been obtained from very small numbers of organisms after as little as 10 minutes of incubation and a total elapsed time of 25 minutes. However, there have been times when the determination for small numbers of cells has taken several hours. More research is needed in order to determine the reasons for and to attempt to control this biological vagary.

Variations in quantities of $C^{14}O_2$ produced per cell per hour pose a problem in quantitizing the test. If the rate of CO_2 evolved by coliform organisms can be made reasonably uniform, it will be

possible to obtain a quantitive determination without resorting to testing numerous replicates of various dilutions as is required in the standard method test. A significant factor in the variability of CO_2 production rates may be incorporation or retention of the gas by cells reproducing or preparing to reproduce. The use of poisons to prevent the incorporation or retention is being investigated.

Since the project began, the amount of 1-C^{14} required per test has been reduced to the point where the radioisotope method can compete economically with the standard method. Radioactivity counting equipment required by the method represents an initial investment of approximately one thousand dollars. The technic is simple and safe. Two important items, the Robinson gas-flow counter and the 1-C^{14} lactose, are not commercially available at the present time.

The possibility exists that the method can be made confirmatory for coliform organisms. Use of radioisotope technics should also yield rapid determinations for other types of organisms by proper design of the test methods and media. In addition to water bacteriology, application of such methods should be practical in milk and food quality control and other public health work. It is also believed that a rapid method may be developed for total bacteria determinations. The latter would have important civil defense implications as a means for detecting bacteriological warfare.

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