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Use of Radioactive Culture Media

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SINCE the use of radioactive culture media in a rapid presumptive test for coliform organisms was first reported in the Journal (*1*), significant changes in method and equipment have been made. Experiments have been conducted with several different culture media, and work has begun toward a rapid confirmed test.

Before proceeding with a discussion of current work, however, a brief review of the general principles of the method might be of assistance to those unfamiliar with it. The rapid presumptive test relies upon the same criteria for the presumptive identification of coliform organisms as does the one outlined in *Standard Methods* (*2*)—namely, the ability of coliform organisms to ferment lactose with the production of gas. The difference between the two tests lies in the means of detecting the gas. In the *Standard Methods* procedure, sufficient gas produced by the organisms must accumulate until a visible bubble is evident. This requires from 24 to 48 hr of incubation. If the gas is made radioactive, however, extremely small quantities of it can be detected. The time required for the determination is thus greatly reduced.

A large portion of the gas produced from lactose by coliform organisms is carbon dioxide. By substituting radioactive carbon in the lactose, the carbon dioxide produced by the organisms can be made radioactive. Thus, if a sample of unidentified bacteria is placed in standard lactose broth (*2*) prepared with radioactive lactose and then incubated at 37°C, the evolution of radioactive carbon dioxide constitutes a positive presumptive test for coliform organisms. In actual practice, the amount of radioactivity evolved from a test sample is compared to that evolved from a sterile control. The comparison is necessary because a small quantity of radioactive carbon dioxide is evolved from sterile radioactive lactose. If the radioactivity evolved by the test sample is significantly higher than that evolved by the sterile control, the test is positive.

Presumptive Test

The unit shown in Fig. 1 is the combined bacteriological culture and carbon dioxide trapping device developed for the rapid test. The sample of water to be tested is filtered through a small membrane filter. The filter is then placed in the metal cup or planchet

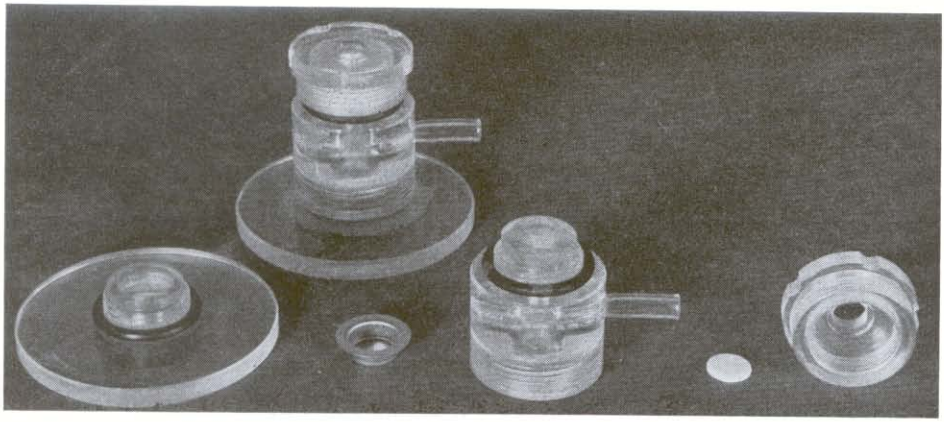

Fig. 1. Combination Culture and $C^{14}O_2$ Collection Unit

The components of the unit (foreground) are shown, left to right, in the order in which they are assembled—base, culture cup, middle section, paper pad, and top section. A fully assembled unit is shown slightly behind the parts.

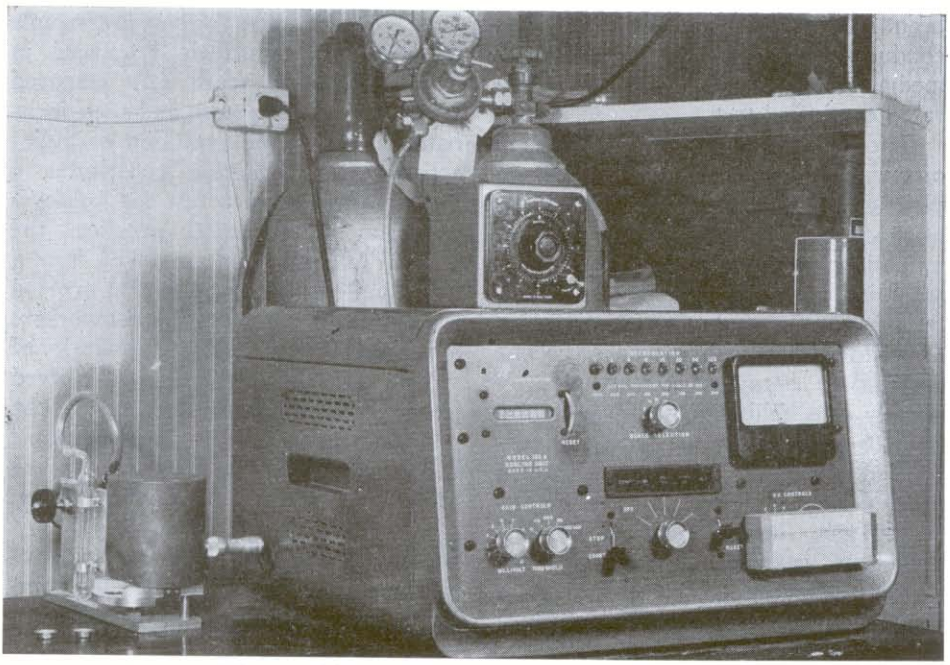

Fig. 2. Counter and Scaler

The modified Robinson gas-flow counter is shown to the left, with two planchets ready to be placed in it. The scaler is to the right, and the tank supplying the counting gas is to the rear.

which fits into the base section of the plastic unit. A pipet is used to place 0.1 ml of the radioactive lactose broth on the filter. The middle section of the unit, consisting of a chimney with an air tube projected into it, is then screwed tightly onto the base. A paper pad is fitted into the top section and is wetted with one drop of a saturated solution of barium hydroxide. The top section is then screwed on, thus completing the assembly of the unit, and the base of the unit is immersed in a 37°C water bath. Filtered air from the laboratory compressed air line is introduced into the air tube of the middle section. The aeration rate is maintained at approximately 5 ml per minute by bubbling the air through mineral oil. The portion of the tube traversing the chimney has three small holes which direct the air downward to the broth. The air sweeps the surface of the broth, entraining any carbon dioxide that had diffused to the surface, and carrying it up the chimney. The carbon dioxide is deposited on the pad as barium carbonate. The remaining gases pass through the pad. After the desired interval has elapsed, the top section is removed and the pad is placed, exposed side up, in a small planchet. The pad is dried by placing the planchet under a heat lamp for 10 min. The planchet containing the pad is then placed into the modified Robinson gas-flow counter, shown in Fig. 2, and measured for radioactivity. A counting period of 5 min has been adopted. The culture and collection apparatus currently in use is shown in Fig. 3. A manifold supplies air, metered through the vertical test tubes of mineral oil, to as many as sixteen units simultaneously.

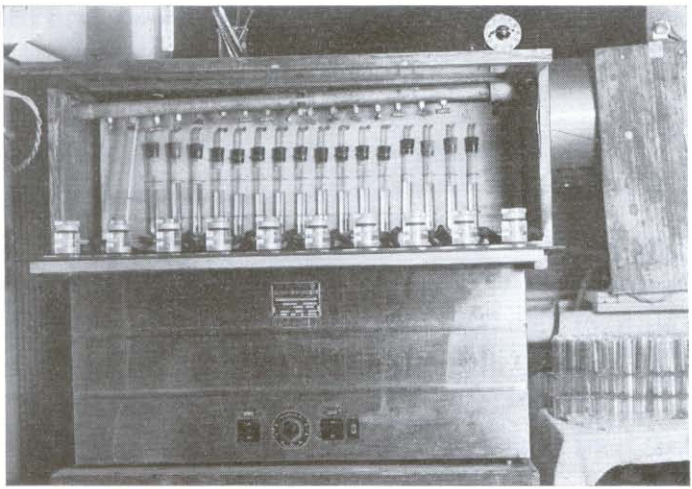

Fig. 3. Bank of Units

A manifold supplies laboratory air through the needle valves to the test tube bubble meters, and thence to the units. Ten units are shown on the work shelf prior to partial immersion in the water bath for 37°C incubation. The apparatus can handle sixteen units simultaneously.

The radioactive lactose is 1-C^{14} lactose synthesized at the National Bureau of Standards (*3*). It has been obtained with specific activities varying from 1.79 to 5.46 microcuries (μc) per milligram. Thus, when lactose with a specific activity of 5.46 μc per milligram is used, 0.1 ml of a 0.5 per cent solution—the standard method concentration—contains a total of 2.73 μc which is the amount of radioactivity used per test. Current AEC regulations permit purchase of 500 μc of C^{14} without requiring the purchaser to obtain a license or equip the laboratory in any special fashion. The use of 1-C^{14} lactose in the rapid presumptive test for coliform organisms was recently described (*4*) in the literature.

Broth Strength Variances

Quantities of a 1-C^{14}-lactose broth were prepared in various strengths to determine if increased concentrations of available lactose would increase CO_2 production by the organisms. In a massive inoculation experiment, *Esch. coli* were incubated in nutrient broth at room temperature for 16 hr. A 0.5-ml portion of the culture was transferred to 5.0 ml sterile tap water and held at room temperature for 24 hr. One drop of this suspension (10^6 to 10^8 cells) was used to inoculate each of the presumptive test cultures. The method used has already been described (*4*). Concentrations of 0.5, 1, 2, and 3 per cent 1-C^{14} lactose, each in three replicates, were run for 1 hr. The results are shown in Table 1. The effect of increased lactose concentration is readily apparent in the table. In the interests of conservation of isotope, the concentration effect has not been pursued to its maximum.

TABLE 1

Effect of Concentration of 1-C^{14} Lactose on $C^{14}O_2$ Production*

Time After Inoculation *min*	Radioactivity of Evolved $C^{14}O_2$ *cpm*†											
	Concentration—*0.5%*‡			Concentration—*1%*‡			Concentration—*2%*‡			Concentration—*3%*‡		
15	1,041	1,107	843	1,304	1,610	2,129	3,495	1,838	3,231	3,471	3,280	3,014
30	2,346	2,222	1,913	2,573	2,533	2,416	5,900	5,735	5,515	5,629	5,260	4,424
45	442	377	362	422	311	433	2,132	1,656	1,460	4,947	4,341	2,887
60	84	100	102	103	79	110	443	371	417	3,337	4,210	3,639
Total	3,913	3,806	3,220	4,402	4,533	5,088	11,970	9,600	10,623	17,384	17,091	13,964
60-min Average	3,646			4,674			10,731			16,146		

* Produced with equally massive *Esch. coli* inocula.
† Radioactivity was measured above a background of 7 cpm.
‡ Three replicates of the same concentration were used.

To test the concentration effect on low numbers of *Esch. coli* cells, a suspension was prepared essentially as that just described. Prior to inoculation, the suspension was serially diluted 10^7 fold. One drop of this suspension was inoculated into each of the test cultures. Plate count determinations revealed the presence of approximately 5 cells per test. Concentrations of 3, 4, and 5 per cent 1-C^{14} lactose, each in

three replicates, were run for 1 hr. Three sterile controls were run using 5 per cent 1-C^{14} lactose concentration. The highest concentration was used for the control since it would produce the highest nonmetabolic evolution of $C^{14}O_2$. Thus all test to control comparisons would be conservative. The results are shown in Table 2, in which the effect of concentration is again evident. A total of eight 1-hr runs, containing 61 inoculated replicates, was made in which the concentration of 1-C^{14} lactose varied between 1 and 5 per cent, and the size of inocula varied between 5 and 200 lag-phase cells. Forty-two of the replicates produced quantities of $C^{14}O_2$ significantly above that of their sterile controls. The nineteen replicates that did not produce positive results contained concentrations of 1-C^{14} lactose of 2 per cent or less.

TABLE 2

Effect of Concentration of 1-C^{14} Lactose on $C^{14}O_2$ Production*

Time After Inoculation *min*	Radioactivity of Evolved $C^{14}O_2$ *cpm*†											
	Inoculated Tests									Sterile Controls		
	Concentration—*3%*‡			Concentration—*4%*‡			Concentration—*5%*‡			Concentration—*5%*‡		
15	69	59	65	82	74	95	133	102	116	77	53	89
30	77	67	63	84	65	83	115	93	101	64	37	65
45	61	45	35	53	46	55	67	48	65	39	22	36
60	61	38	40	53	38	49	71	50	71	41	22	33
Total	268	209	203	272	223	282	386	293	353	221	134	223
60-min Average	227			259			344			193		

* Produced with approximately 5 *Esch. coli* cells per test in 1-C^{14} Lactose broth.
† Radioactivity was measured above a background of 7 cpm.
‡ Three replicates of the same concentration were used.

pH Effect

The influence of pH on the test has been found to be pronounced from a biological and a chemical point of view. Inoculation into 1-C^{14} lactose broth at pH 8 has been found to produce the greatest quantity of metabolic CO_2. Increases of as much as 100 per cent over quantities of CO_2 produced by equal inocula at initial pH 6 or 7 have been found when massive inocula were used. At the end of the 1-hr run, the final pH in such tests has been found to be acid due to the metabolism of the organisms. Massive cultures thus enjoy the initial advantage of biological activity at pH 8 and the subsequent chemical advantage of the acid pH in liberating from solution the CO_2 produced. The chemical effect, however, works against light inocula tests since there are insufficient organisms present to lower the pH to the acid range. Much of the CO_2 produced by the organisms thus remains in solution as the bicarbonate ion. It may be possible to allow the cultures to metabolize for 1 hr, or some desired period, and then to add acid to liberate the dissolved CO_2 for subsequent immediate collection.

Confirmatory Media

The *Standard Methods* (*2*) procedure requires that the confirmed test for coliform organisms be made subsequent to the presumptive test. The confirmed test cannot be made directly, because, in addition to being inhibitory to noncoliform organisms, the confirmatory media are also somewhat inhibitory to coliform organisms. There are, usually insufficient coliform organisms present in a water sample to overcome or survive this effect. The organisms are thus cultured to high densities in the fairly selective presumptive medium and a massive inoculum is then transferred to the confirmatory medium. It seemed possible that a small inoculum of coliform organisms might survive and respire in the confirmatory media long enough to be detected by the radioisotope method. If this were the case, a rapid confirmed test might be made directly from the water sample. In this event, time could be saved by shifting efforts to refine the method from the presumptive to confirmatory media. To explore this possibility, tests were made using various confirmatory media incorporating radioactive compounds.

A crystal violet broth containing 1-C^{14} lactose, a brilliant green bile broth containing 1-C^{14} lactose, and a formate ricinoleate broth containing C^{14} formate were prepared. Each of the media was made in accordance with the *Standard Methods* procedure (*2*). Exponentially growing *Esch. coli* cells inoculated into these media were able to survive sufficiently long to yield $C^{14}O_2$ in excess of the sterile control levels. Approximately 100 cells inoculated into three replicate crystal violet broth cultures produced an average of 48 counts per minute (cpm) on the $C^{14}O_2$ collection pads in 1 hr, as opposed to an average of 38 cpm for three sterile controls. In a serial dilution test of *Esch. coli* in C^{14} formate ricinoleate broth, the cutoff point for statistical significance within a 1-hr test period occurred at the dilution containing approximately 18,000 cells, averaging 179 cpm for test portions compared to an average of 135 cpm for the controls. The nonmetabolic evolution of $C^{14}O_2$ was higher from the formate than from 1-C^{14} lactose. The formate, however, was of higher specific activity, containing 35.8 μc per test portion. Results with exponentially growing cells in 1-C^{14} lactose brilliant green bile broth have shown sensitivity to approximately 15,000 cells in 1 hr. As work has just begun with the confirmatory media, no results are yet available with lag-phase cells.

The ability of *Esch. coli* cells to produce detectable quantities of $C^{14}O_2$ is only one aspect of producing a rapid confirmed test. It must also be shown that lactose fermenting noncoliform organisms will not produce detectable quantities of $C^{14}O_2$ in the confirmatory media. It seems quite probable that lactose fermenting noncoliform organisms might survive the inhibitor sufficiently long to do this. If this is found to be true, it may be possible to delay the $C^{14}O_2$ collection phase of the test sufficiently to allow the noncoliform organisms to die, flush all $C^{14}O_2$ out of the culture, and begin collection in the normal manner.

Nonmetabolic $C^{14}O_2$

The evolution of radioactive carbon dioxide from sterile control portions of radioactive media continues to be the most troublesome difficulty. At times, the level remains essentially at 0 cpm for several consecutive runs, and it

seems the problem is solved. The next run, however, will show that the level has unaccountably soared to 50 or 100 cpm. While being relatively consistent within each run, the unexplained variability of the level of nonmetabolic $C^{14}O_2$ emitted from run to run makes it necessary to include sterile controls in each run. This same defect frequently impairs the sensitivity of the test by adding to the level of radioactivity required for statistical significance of the test results. Work on methods to reduce and control this phenomenon is continuing. The most encouraging development in this direction is the determination that the $C^{14}O_2$ is, at least primarily, produced as the result of a chemical reaction, rather than as a direct product of radioactive disintegration of the lactose. This has been shown by studies of nonmetabolic evolution of $C^{14}O_2$ as a function of temperature of sterile 1-C^{14} lactose broth. In one instance, three sterile 0.1-ml portions of 1-C^{14} lactose were taken from a common pool. Each portion was placed in a separate detection unit and collection of CO_2 begun. The only difference between the three portions lay in the temperatures, which were maintained at 10, 25, and 37°C, respectively. At the end of $\frac{1}{2}$ hr, the collecting pads revealed suprabackground radioactivity levels of 2.2 cpm, 10.6 cpm, and 16.2 cpm, respectively. These are greater differences than can be accounted for by increased solubility of CO_2 at lower temperatures. Other sterile tests made at approximately 5–7°C have yielded noise levels of 0 cpm for 1-hr periods while high levels were obtained from replicates at 37°C. As radioactive decay rates cannot be affected in any manner by temperature change, these results support the conclusion stated. The process may begin with radioactive degradation products, but then goes through one or more chemical reactions before $C^{14}O_2$ is produced. This conclusion was recently confirmed by other workers (*5*) dealing with tastes produced by irradiation of foods. It might, therefore, be possible to incorporate a reagent into the medium that would arrest the stepwise reaction before the $C^{14}O_2$ is produced.

Summary

The rapid method for the presumptive determination of coliform organisms has been considerably refined since first reported. The principal changes have been in the development of a compact culture and CO_2 trapping unit, and the reduction in the quantity of isotope used per test. Other results of current experiments have shown that:

1. Concentration of lactose in lactose broth has a distinct direct effect upon CO_2 production by *Esch coli*.
2. The pH exerts a pronounced biological and chemical effect upon the test. At pH 8, the organisms produce maximum quantities of CO_2 during the 1-hr test. When the broth is on the alkaline side, however, much of the CO_2 remains in solution in the form of the bicarbonate ion. Addition of a drop of acid at the end of the run might be used to liberate the CO_2.
3. It may be possible to make direct confirmatory determinations by the radioactive method.
4. The nonmetabolic evolution of $C^{14}O_2$ from the sterile 1-C^{14} lactose is the chief immediate problem demanding attention in the development of the test.

Conclusions

The radioisotope method has been found to be capable of remarkable sen-

sitivity to extremely small numbers of *Esch. coli* cells. Results, however, are not uniform. It is believed this is due to some yet unrecognized environmental factors which are not controlled. In addition, emission of $C^{14}O_2$ from the sterile control presents a difficulty of nonbiological origin. If these problems can be overcome, however, it will be possible to obtain results of presumptive, and perhaps, confirmed, coliform tests in 1 or, at most, several hours.

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