Second United Nations
International Conference
on the Peaceful Uses of
Atomic Energy

A/CONF.15/P/820
U.S.A.
June 1958

ORIGINAL: ENGLISH

RAPID BACTERIOLOGICAL DETECTION AND IDENTIFICATION

by

G. V. Levin, V. R. Harrison, W. C. Hess,
A. H. Heim, and V. L. Stauss*

The length of time required for completion of bacteriological analysis by current methods makes it impossible to determine the quality of our drinking water in advance of consuming it. After a bacteriological test is initiated, 48 to 72 hours must elapse before the results can be obtained by standard methods (1). Thus, we can only know what the quality of the water was, never what it is.

The sanitary index of drinking water quality is the coliform organism which is universally present in sewage or contamination therefrom.

All accepted methods for detecting these organisms require sufficient cell multiplication to yield direct visual evidence of colonies or gas production. The standard method requires that a single coliform bacterium must give rise to a population of 1.7×10^8 cells before sufficient gas can be evolved to produce a visible bubble. The membrane filter technique which is now accepted in various jurisdictions, and will shortly become a standard method, shortens the elapsed time to 22 hours, but this is also a dangerously long period of ignorance.

To overcome the disadvantages of current methods for the bacteriological examination of water, it is necessary to develop a method capable of measuring a parameter of sufficiently minute dimensions to indicate the presence of a small number of cells with little or no time-loss for incubation and multiplication. Furthermore, the method must be simple enough to be used as a routine laboratory tool.

* Sanitary Engineer, Resources Research, Inc., Wash., D.C.; Research Assistant, School of Medicine, Georgetown University, Wash., D.C.; Asst. Dean of Research and Prof. of Biological Chemistry, School of Medicine, Georgetown University, Wash., D.C.; Assistant Professor of Biological Chemistry, School of Medicine, Georgetown University, Wash., D.C.; and Research Assistant, School of Medicine, Georgetown University, Wash., D.C.; respectively.

Radioisotope detection instruments are capable of sensing particles many trillions of times smaller than a bacterium. If bacter would incorporate unstable atoms, radiation from the harvested cells o from their metabolic products would indicate the presence of the bacte Isotopes have been used to study metabolic pathways of various compoun utilized by large populations of bacteria (2). If selected isotopes i quantities sufficient for subsequent detection were utilized by small numbers of bacteria, a rapid method for bacterial determination might result.

This paper presents the several procedures that have evolved since the investigation started in 1955, and suggests that the original idea will produce a method that can detect and confirm the presence of small numbers of coliform bacteria within several hours.

EXPERIMENTAL

The standard method for the presumptive identification of coliform organisms employs a culture medium containing lactose. A positive test results when a visible bubble of gas is produced from the lactose by the growing cells. If the lactose is labeled with C^{14}, then the carbon dioxide produced should include some of the labeled carbon that could be detected and measured. Lactose labeled with C^{14} in the number one carbon, obtained from the National Bureau of Standards (3), was used to prepare standard lactose broth (1). Test organisms were inoculated into the broth and incubated with aeration. The carbon dioxide in the exhaust air was collected and examined for radioactivity after short periods of time. The collection of carbon dioxide was initially achieved by passing the exhaust gas through a saturated solut of barium hydroxide. The barium carbonate precipitated in this fashion was filtered, dried, and counted for radioactivity.

In a typical experiment 10 ml. of standard lactose broth containing 0.5 per cent 1-C^{14} lactose (1.79 μc/mg.) were inoculated with 26 Escherichia coli cells, as subsequently determined by plate counts of the inoculum suspension. The radioactivity of the gas evolved by the cells was determined hourly. When the total activity appreciably exceeded that evolved from a sterile control, it was considered significant for a positive determination. Two hours produced sufficient activity in some of the early tests. It was found, however, that a signifi amount of the radioactive barium carbonate remained in solution. In o to eliminate this source of error and to reduce the number of steps, a modification was made in the method of collecting the carbon dioxide. The exhaust gas was passed through a paper fiber pad moistened with a saturated solution of barium hydroxide. At selected intervals the pad removed from its holder, dried under an infra-red lamp, and the activi determined. The results of such an experiment are shown in Table I. inoculum, consisting of approximately 125 E. coli cells, produced a po tive presumptive test in one hour.

TABLE I

Presumptive Test of Sample Containing Approximately 125 E. coli

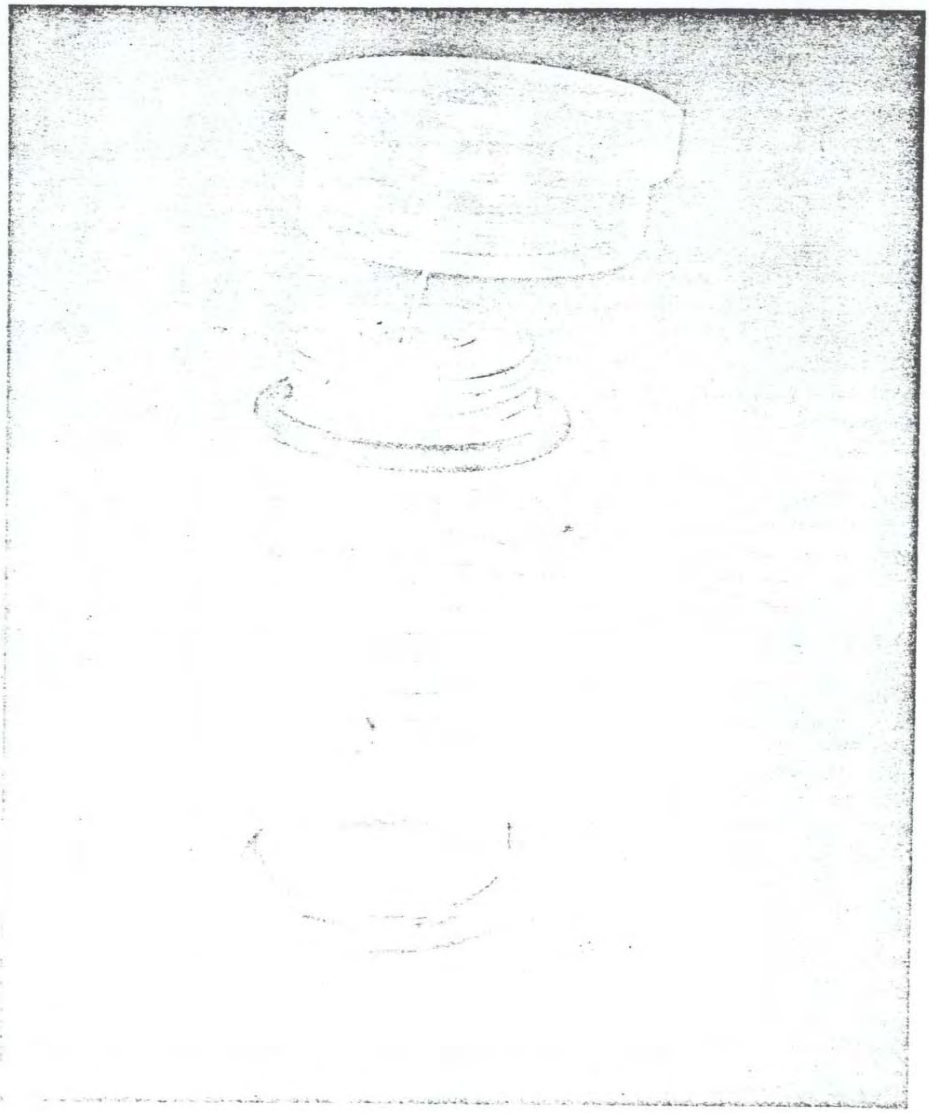
Time hr.	Radioactivity of Test*—cpm		Radioactivity of Control*—cpm	
	Increment	Cumulative	Increment	Cumulative
0	106		99	
1	172	172 †	67	67
2	309	481	38	105
3	1,154	1,635	36	141
4	4,075	5,710	36	177
5	12,579	18,289	27	204

* Radioactivity measured above a background of 21 counts per minute.

† Point of presumptive determination.

River water taken from a sewage-contaminated area was tested to determine the practical applicability of the method. An aliquot of the water sample (55.5 ml.) was filtered through a membrane filter. The filter was removed and immersed in a test tube containing 5 ml. of C^{14}-standard lactose broth. The same procedure was employed for incubation and determination as described for the experiment cited in Table I. The standard method of water analysis was run at the same time. At the end of 10 hourly inspections, no gas production was observed in the standard test; at the end of 24 hours, gas production was noted. In the radioactive test experiments, as shown in Table II, a positive test was noted in 30 minutes.

Figure 1.



Exploded view of combination culturing and carbon dioxide trapping unit. (The wire is for the purpose of positioning the unit components for the photograph.)

TABLE II

Radioisotope Test on Raw River Water

Time min.	Radioactivity of Test*—cpm		Radioactivity of Control*—cpm	
	Increment	Cumulative	Increment	Cumulative
30	1,598	1,598 †	28	28
60	1,578	3,176	23	51
95	2,428	5,604	18	69

* Radioactivity measured above a background of 21 counts per minute.

† Point of presumptive determination.

While these results were gratifying, a practical test had not yet been achieved. The use of 5 ml. of culture media required an amount of radioactive lactose that cost-wise was prohibitive. In an effort to reduce the volume of medium, a combined bacteriological culture and carbon dioxide-trapping device was developed as shown in Figure 1. The water sample to be tested is filtered through a small membrane filter. The filter is then placed in the metal cup or planchet which fits into the base section of the plastic unit. A pipette 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 fitted tightly to the base. A paper pad is placed into the top section and is moistened with one drop of a saturated solution of barium hydroxide. The top section is put 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. The portion of the tube traversing the chimney has three small holes which direct the air downward to 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, dried, and counted for radioactivity.

In order to determine the optimum concentration of lactose for the maximum production of radioactive carbon dioxide, various dilutions of lactose broth were prepared and inoculated with small numbers of cells. Concentrations of 3, 4, and 5 per cent 1-C^{14} lactose, each in three replicates, were inoculated with approximately 5 E. coli cells as determined by plate counts. Table III gives the counts per minute obtained for periods of time up to 60 minutes with the several concentrations. The maximum number of counts was obtained with 5 per cent lactose but all three concentrations gave positive tests in 60 minutes.

TABLE III

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%			Concentration5%		
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 E. coli cells per test in 1-C^{14} lactose broth.

† Radioactivity was measured above a background of 7 cpm.

‡ Three replicates of each concentration were used.

At this stage of the experiments it became necessary to consider the metabolic state of the organisms. There are two general conditions that may obtain: the organisms may be actively reproducing, a condition known as the logarithmic growth phase, or the organisms may be in a state of comparative rest with reproduction at a minimum and metabolism sufficient only to maintain life, a condition known as the lag

state. Organisms present in a sample of drinking water or raw water are likely to be in lag phase and thus tests on this state would more closely approximate actual conditions under which the method would be used.

Lag was induced by chilling a suspension of E. coli cells, made from an 18-hour nutrient broth culture, for three hours at 5° C. 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 μc per mg. Thus 7.2 μc were contained in each planchet. To each of three of the planchets 0.1 ml. of the chilled bacterial suspension was added. The fourth planchet 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 incubated at 37° C.; aeration was at the rate of 3 bubbles per second. The barium hydroxide treated pads were replaced every 10 minutes. Table IV shows the results obtained with various numbers of cells. In ten minutes the amount of radioactive carbon dioxide collected from each of the cultures was sufficient to determine that the presumptive test for coliform organisms was positive.

TABLE IV

Presumptive Test for E. Coli in Lag Phase

Time (Minutes)	Radioactivity*--(cpm)							
	Approximate No. of Cells							
	115		40		20		Control	
	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 min.

† Point of presumptive test determination.

Contrary to expectations, the time required for positive results was consistently longer when the E. coli were in exponential growth than when they were in lag phase. 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. Immediately upon preparation, 10 ml. of each suspension were filtered through a membrane filter. Each filter was then immersed in a planchet containing 0.9 of 1-C^{14} lactose broth. A fourth 0.9 ml. portion of the broth was maintained as a sterile control. The results are given in Table V. Comparison of the results in Table V with those in Table IV reveals the difference in rate of evolution of gas between lag-phase and exponential-phase organisms. One hypothesis that can relate these disparate results is that reproducing cells and cells preparing to reproduce conserve large quantities of carbon dioxide for use in building cellular material. Lag phase cells might not exert such a carbon dioxide demand.

TABLE V

Presumptive Test for E. coli in Exponential Growth

Time (Minutes)	Radioactivity*--(cpm)							
	Approximate No. of Cells							
	13,000		4,800		1,680		Control	
	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	1168	51	188	32	51	15	93
210	1847	3015	85	273	50	101	24	117
240	2600	5615	120	393	102	203†	16	133

* Radioactivity is measured above a background of 23 cpm.

†Point of presumptive test determination.

While coliform organisms are the index of water contamination, they are not the only bacteria that will produce carbon dioxide from lactose. Hence the evolution of gas constitutes only presumptive evidence for the presence of coliform organisms. Accordingly, the standard test employing the lactose medium is known as a presumptive test. To eliminate the other possible gas producers, a confirmed test is employed as a second stage in the standard analysis. The media used for a confirmed test contains, in addition to a carbon source, some inhibitor of noncoliform organisms. One of the media used in the confirmed test is formate ricinoleate broth (1) which contains both lactose and formate. Formate containing C^{14} can be obtained at a cost far less than that of 1-C^{14} lactose, and a series of experiments was designed to investigate the use of C^{14} formate in the procedures previously described. Another medium used for a confirmed test is lactose broth with brilliant green bile added.

A series of experiments utilizing lactose and sodium formate in nutrient broth to determine the optimum concentration of each which would result in maximum gas production by E. coli was performed. Fermentation tubes were uniformly inoculated with light suspensions of E. coli at various concentrations of nonradioactive lactose and formate. It was clearly indicated that the addition of sodium formate to lactose broth, in the manner of formate ricinoleate medium, resulted in enhanced gas production by E. coli cells over that obtained from lactose medium alone.

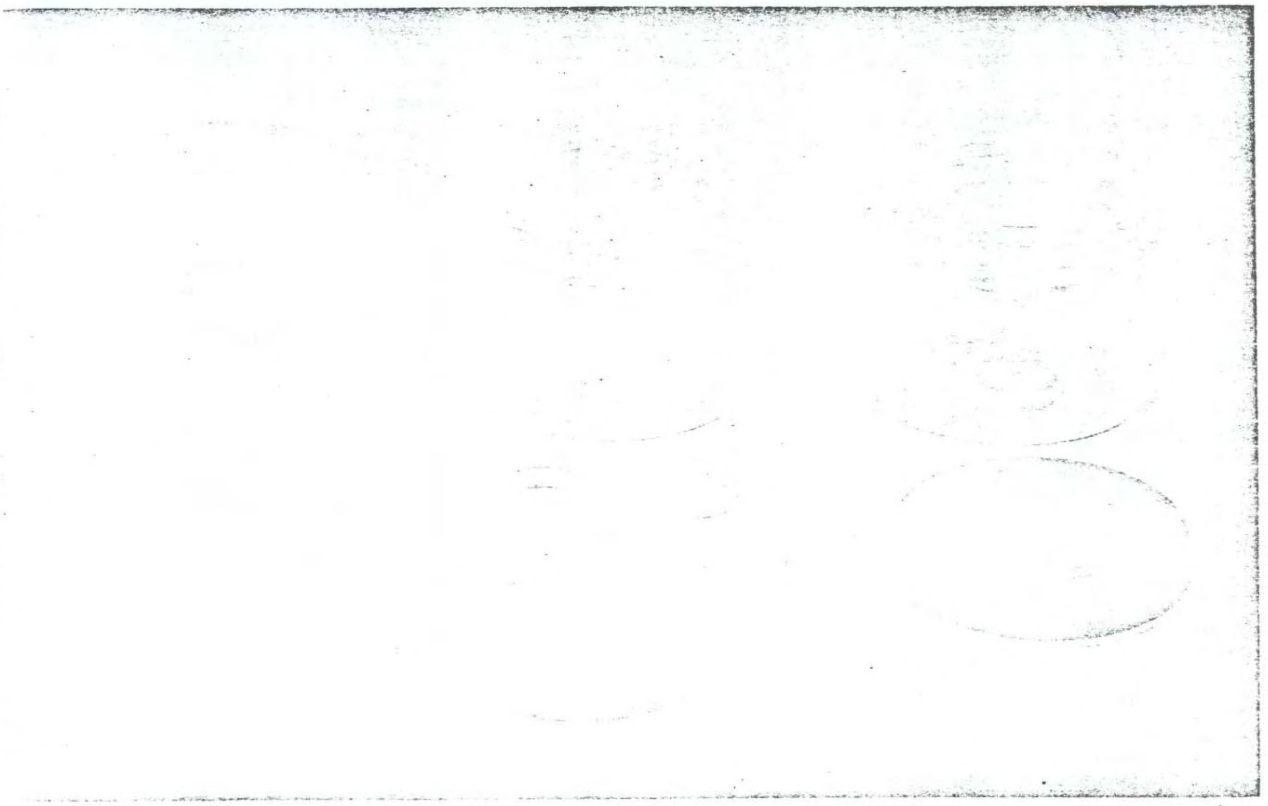
The work of Roberts et al. (4) demonstrated that C^{14} formate when metabolized by E. coli resulted in the following distribution of C^{14}: $C^{14}O_2$ - 86%; incorporated in cells - 12%; remaining in medium - 1.9%. This distribution of the C^{14} suggested excellent possibilities for the utilization of C^{14} formate in a basal medium for the detection of coliform bacteria.

A series of experiments were performed utilizing C^{14} formate in concentrations from 1% to .0025%. By substituting brilliant green for sodium ricinoleate as the noncoliform inhibitor, an attempt was made to develop a direct confirmed test for the detection of the coliform bacteria. Two per cent brilliant green lactose bile broth was employed as the base medium.

The combination culturing and $C^{14}O_2$ collecting device previously described was superceded by a new collection method which, after numerous comparative determinations, has demonstrated increased collection efficiency as well as operational simplification.

Two procedures are utilized: (1) where large concentrations of cells are present and (2) where there are low numbers of cells present.

Figure 2.



Two-ounce jar assemblies used to incubate cultures and effect transfer of CO_2 evolved from culture planchet to barium hydroxide planchet by diffusion.

Procedure I: The culture planchet containing 0.1 ml. of media and inoculum is placed in a 2-oz. screwcap jar together with a planchet containing three drops of a saturated solution of barium hydroxide and one drop of 0.5% sucrose. The sucrose aids the spreading of the barium carbonate as it dries. The cap is secured and the jar placed in an incubator for a period of one hour or less. The barium hydroxide planchet is then removed, dried, and counted for radioactivity.

Procedure II: The culture planchet containing 0.1 ml. media and inoculum is placed in a 2-oz. screwcap jar. The lid is secured and the jar is incubated for three hours at 37° C. The cap is then removed and a planchet containing a saturated solution of barium hydroxide and sucrose, as in Procedure I, is placed in the jar with the culture planchet. The lid is again secured and the jar incubated an additional hour. The cap is removed; the barium hydroxide planchet taken out, dried, and counted for radioactivity.

Experimental evidence has demonstrated that exposure of the culture planchet to barium hydroxide for the entire incubation period of the extended test results in a marked diminution of $C^{14}O_2$ evolved. This is not a problem in obtaining qualitative results with large inocula in the one hour test.

The following series of tests were performed utilizing the collection method described. Figure 2 illustrates the method. Procedure: C^{14} formate was utilized in the concentrations indicated in a 2 per cent brilliant green lactose bile broth. A dilute cell suspension of E. coli was held at room temperature in a distilled water blank to induce lag conditions. A loop of the cell suspension, (containing approximately 140,000 cells as determined by replicate plate counts made at the time of inoculation), was placed in three replicate culture planchets containing 0.1 ml. of the tagged media. Similarly, a loop of sterile distilled water was placed in three replicates containing sterile radioactive media as controls. Each inoculated planchet was incubated in accordance with Procedure I.

TABLE VI

C^{14} Formate Conc. %	Inoculated Sample (Avg. cpm above control)
1.0	36
0.5	89
0.1	126

The results shown in Table VI indicated that the C^{14} formate in the higher concentrations was toxic to the organisms. Another test employing C^{14} formate in a lower concentration was performed as follows:

Procedure: C^{14} formate was utilized in the concentrations indicated in 2% brilliant green lactose bile broth. A dilute suspension of E. coli cells in distilled water was chilled three hours prior to use to induce lag. One loop of various dilutions was added to three replicate planchets containing 0.1 ml. of tagged medium. The test procedure was the same as that which produced the data in Table VI. The results are given in Table VII.

TABLE VII

No. of Cells	Results with 0.1% C^{14} formate (Avg. cpm above control)	Results with 0.05% C^{14} formate (Avg. cpm above control)
21,500	39	41
11,500	24	37
5,600	9	12
1,740	6	2

Results of this test indicated that the use of still lower C^{14} formate concentrations was necessary to determine clearly the point at which minimum toxicity and maximum $C^{14}O_2$ production occurred.

The next series of tests utilized the method previously described as "Procedure II" because lower cell concentrations were used.

Procedure: C^{14} formate was employed in 2% brilliant green lactose bile broth in various concentrations. Dilute suspensions of E. coli cells were chilled three hours prior to use. Culture planchets of brilliant green bile lactose broth containing various concentrations of radioactive formate were inoculated in triplicate. Three sterile controls were included in each set. Procedure II was followed.

TABLE VIII

No. of Cells	Results with _% C^{14} NaFormate/2% BGBLB (Avg. cpm above control)			
	.05	.01	.005	.0025
4,800	5,300	5,450	2,187	747
2,450	3,100	2,410	1,016	333
1,177	962	736	332	139
577	352	371	245	57
360	---	253	115	43

The results are given in Table VIII. The optimum concentration of C^{14} formate was indicated as being between 0.05% and 0.01%. To minimize any possible toxic manifestations, 0.01% was selected as the concentration of choice to be employed in the next series of experiments which were designed to investigate the quantitative aspects of the test. Procedure: 0.01% C^{14} formate in 2% brilliant green bile lactose broth was employed for the entire series of tests. The cells employed were suspensions of 20-24 hour E. coli cultures. The suspensions were chilled for 2 or 3 hours just prior to inoculation. The inoculum consisted of a single loopful of the desired cell suspension per planchet containing 0.1 ml. of tagged media. Sterile controls containing a loop of sterile distilled water were included with each test. A system of three replicates for each cell dilution and control was used throughout. Thus the data presented in Table IX represents three determinations at each cell dilution in each test. Approximate cell numbers were determined by triplicate plate counts in each case. Procedure II was followed in all cases.

TABLE IX

Correlation Between Numbers of E. coli cells and Evolved Radioactivity

	CPM* Produced by Cell Population Range Indicated								
	Number of Cells								
	3,200-6,400	1,600-3,200	800-1,600	400-800	200-400	100-200	50-100	25-50	1-25
	5,450	2,400	762	342					
			985	488	358				
			480	285	149				
			520	247	117				
		1,871	771	321	167	66	37	15	
			669	254	109	36	14		
	6,262	4,000	1,676	755	245	105	40	28	15
		1,621	858	472	224	96	57	24	16
		1,336	768	298	164	93	39	24	
	6,060	2,283	1,373	673	391	135	52	37	
	6,271	1,820	906	449	224	155	89	37	11
					106		31		7
	4,290	1,625	792	400	181	94	64	27	
Avg.	5,667	2,119	880	415	203	98	47	27	12
Std. Deviation	± 839	± 841	±340	±163	± 92	±37	±21	± 7	± 4

* Average of 3 replicates minus sterile controls.

Each horizontal row of figures represents a separate test run for which all data obtained are presented.

The data have been presented in cell population ranges which permit internal variations of one-third of the average population for the range. Normal plating procedures by which the cell numbers were determined generally produce variations of as much as ten per cent in replicates. A special consideration compounds this variation for the radioactive test. In effect, the test attempts to correlate gas production by cells incubated four hours with the numbers of visible colonies produced by cells incubated 24 hours. Some of the cells in the suspensions inoculated might live long enough to produce radioactive gas in the rapid test, but their counterparts in the plate count test might not survive long enough to start colonies. The problem of knowing how many cells have been inoculated into a radioactive test culture is an innately difficult one to solve. While a greater degree of accuracy in determining the numbers of cells detected by the radioactive test is ultimately desirable, the groupings presented enable a gross appraisal of the quantitative prospects for the method. Further tests must be run with various strains of coliform organisms, cultures of differing ages, and noncoliform organisms which might interfere before it can be definitely concluded that a quantitative test with a single test portion is feasible. Although limited in scope, the data in Table IX are encouraging in this respect.

RADIOACTIVITY PRODUCED BY CONTROLS

In earlier work (5, 6, 7), the difficulties associated with non-metabolic emission of $C^{14}O_2$ from the sterile controls has been discussed. These control levels had been fairly unpredictable and frequently interfered with the sensitivity of the test.

The problem of this spurious activity has now been brought under control to the point where it can be routinely reduced to a satisfactory working level. When radioactive lactose was used in the medium, the spurious activity level was reduced by "washing" the medium with CO_2. This was done simply by passing nonradioactive CO_2 over the surface of the medium prior to inoculating it with bacteria. The activity was reduced in this way to 20-40 C.P.M. when collected by the method presently employed.

A change in medium composition to the use of radioactive formate presented the same problem and CO_2 "washing" was again found to reduce the spurious activity level, but it remained higher than was desirable and it was also quite variable. It was found that by preparing the test medium containing 0.01% formate and then mechanically shaking it for a period of 24 hours, the spurious activity can be reduced consistently to 0-5 C.P.M. The stock medium is kept frozen, and shaken for 1 - 2 hours prior to use each time. In this way, a satisfactory working level for controls can be maintained. Consequently, the radioactivity levels produced by the sterile controls have not been reported in the data herein presented.

The cause of the spurious CO_2 evolution is still uncertain. Temperature studies indicate that it is chemical in nature and studies with media treated for the removal of metals suggest the involvement of a metal catalyst. However, the present control methods probably result in an exchange of atmospheric non-radioactive CO_2 with radioactive CO_2 which had been formed in the medium. The rate of formation of radioactive CO_2 is slow enough so that there is no subsequent interference for the duration of the test.

REFERENCES

(1) Standard Methods for the Examination of Water, Sewage and Industrial Wastes, APHA, AWWA, and FSIWA, New York, (10th ed.), (1955).

(2) Cowie, D. B., Bolton, E. T., and Sands, M. K., Sulfur Metabolism in Escherichia Coli, J. Bac., 60:233, (1950).

(3) Frush, H. L. and Isbell, H. S., Synthesis of Lactose 1-C^{14} and Lactobrionic-1-C^{14}-β-Lactose from 3-B-D-Galacto-pyranosyl-α--D-Arabinose, J. Research Nat. Bur. Standards, 50:133, (1953.)

(4) Roberts et al., Studies of Biosynthesis in E. Coli, Carnegie Institution of Washington, Publication No. 607, Washington, D. C., (1957).

(5) Levin, G. V., Harrison, V. R., Hess, W. C., Preliminary Report on a One-Hour Presumptive Test for Coliform Organisms, Jour. Am. Water Works Assn., 48:1, (1956).

(6) Levin, G. V., et al., A Radioisotope Technic for the Rapid Detection of Coliform Organisms, Am. Jour. Pub. Health, 46: 11, (1956).

(7) Levin, G. V., Harrison, V. R., Hess, W. C., Use of Radioactive Culture Media, J. Am. Water Works Assn., 49:8, (1957).