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DETERMINATION OF ANTIMICROBIAL ACTIVITY BY A RADIOISOTOPE METHOD*

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INTRODUCTION

The present report is designed to show the potential of a radioisotope method for (a) rapidly determining antimicrobial activity and (b) studying the interaction between bacteria and an antimicrobial agent. The method is adapted from one used by Levin et al. [1] for the detection of coliform organisms in water.

In principle, the method is essentially the same as any other respirometric technique in which the amount of CO_2 released as a result of metabolism is measured. The method described here consists of seeding a growth medium containing radioactive carbon with bacteria, introducing antimicrobial agents into some of the media, collecting the CO_2 which is evolved, and comparing by means of the tracer the amount of CO_2 to that evolved from antibiotic-free controls. Inhibited cells yield less CO_2 than noninhibited cells.

MATERIALS AND METHODS

Twenty-five-millimeter planchets normally used in standard counting equipment are sterilized and used as culture dishes. Introduced into each culture planchet is 0.8 ml of trypticase-soy broth which contains 0.003% C^{14}-sodium formate (specific activity = 2 mC/mmole, which results in 0.8 μC/planchet). One set of culture planchets is not seeded but serves as a sterile control to determine if any nonmetabolic $C^{14}O_2$ is released from the medium. One set of planchets is seeded with the test organism but is kept free from antibiotics in order to serve as a positive control. Other sets of culture planchets are seeded and have various concentrations of antibiotic added. The final volume of liquid in each planchet is 1.0 ml. The planchets are then incubated at 37 C.

At the end of the desired incubation period, planchets similar to those used for culturing the test organism but containing a paper pad soaked with saturated $Ba(OH)_2$ are inverted over the culture planchets, thereby forming a small collection chamber. The $C^{14}O_2$ diffuses to the pad and is precipitated as $BaCO_3$. The pad containing the labeled $BaCO_3$ is dried directly in the planchet holding it and the amount of radioactivity is determined using standard counting equipment.

Tube-dilution determinations are made concurrently, using the same concentrations of broth, antibiotic, and inoculum.

All of the bacteria thus far investigated have been isolated from patients at Georgetown University Hospital and the numbers shown in the tables are hospital identification numbers.

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Table 1. Effects of Antibiotics on $C^{14}O_2$ Released by a Staphylococcus (7232) in $3\frac{1}{2}$ hr

Antibiotic	Concentration	Counts/min	Tube dilution, 24 hr
None		1147	
Penicillin	1 unit/ml	1969	Growth
	5 units/ml	1784	Growth
	50 units/ml	190	Growth
Chloramphenicol	1 μg/ml	1332	Growth
	10 μg/ml	69	No growth
	50 μg/ml	59	No growth
Tetracycline	1 μg/ml	2486	Growth
	5 μg/ml	2538	Growth
	50 μg/ml	45	No growth
Erythromycin	1 μg/ml	59	No growth
	5 μg/ml	70	No growth
	50 μg/ml	58	No growth

Concentration of cells, 10^{-2} dilution of an 18- to 20-hr culture. Counts/min have background and sterile controls subtracted and are averages of duplicates.

RESULTS

Table 1 presents the results obtained when cells were incubated for 3 hr and the $C^{14}O_2$ was collected subsequently for 30 min. It can be seen that there is complete correlation between the radioisotope method and the tube-dilution determinations. This particular Staphylococcus was completely resistant to penicillin. It exhibited resistance to 1 and 5 μg of tetracycline and only to 1 μg of chloramphenicol. It appeared to be sensitive to all of the concentrations of erythromycin.

The trend of metabolic activity and $C^{14}O_2$ evolution can be observed by collecting the $C^{14}O_2$ over two different time periods (Table 2). It can be seen that the positive control activity shows a considerable increase over the 2-hr interval. The same kind of reaction was observed in all cases where the corresponding

Table 2. Effects of Antibiotics on $C^{14}O_2$ Released by Escherichia coli (10979) in 2 and 4 hr

Antibiotic	Concentration	Counts/min		Tube dilution, 24 hr
		2 hr	4 hr	
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

Concentration of cells, 10^{-2} dilution of an 18- to 20-hr culture. Counts/min have background and sterile controls subtracted and are averages of duplicates.

Table 3. Effects of Antibiotics on $C^{14}O_2$ Released by Proteus (10967) over 6 hr

Antibiotic	Concentration	Counts/min						Tube dilution
		1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	24 hr
None		230	1,500	9,805	27,982	10,839	6,243	
Penicilin	1 unit/ml	200	1,362	8,820	19,766	18,020	3,967	Growth
	5 units/ml	193	1,274	7,552	30,625	14,690	6,403	Growth
	50 units/ml	248	3,441	18,597	28,605	8,545	1,556	Growth
Tetracycline	1 μg/ml	167	401	721	2,613	5,286	17,118	Growth
	5 μg/ml	154	144	311	418	1,781	9,543	Growth
	50 μg/ml	88	37	28	14	11	10	No growth

Concentration of cells, 10^{-2} dilution of 18- to 20-hr culture. Counts/min have background and sterile controls subtracted and are averages of duplicates.

tube dilutions became positive, indicating a lack of sensitivity of the test organism. Decreasing amounts of $C^{14}O_2$ were obtained over the 2-hr interval where the corresponding tube-dilution tubes remained negative.

The length of time over which $C^{14}O_2$ was collected was extended to 6 hr with collections made at 1-hr intervals (Table 3). $C^{14}O_2$ collection was maintained for 15 min between each 1-hr interval. The antibiotic-free control reached a peak at 4 hr and the bacteria exposed to the penicillin all followed the same pattern of $C^{14}O_2$ evolution. The corresponding tubes in the tube-dilution determination were positive when read the following day. The effect of the tetracycline on this particular Proteus culture was different from the effect of penicillin. There apparently was some degree of inhibition even at a concentration of 1 μg, as indicated by the lag in $C^{14}O_2$ evolution, although there was a break at 4 hr with a subsequent lack of sensitivity of the culture. The same effect observed

Table 4. Effects of Antibiotics on $C^{14}O_2$ Released by a Staphylococcus (6018) in 4¼ hr

Antibiotic	Concentration	Counts/min	Tube dilution, 24 hr
None		1450	
Penicillin	1 unit/ml	1189	Growth
	5 units/ml	1333	Growth
	50 units/ml	113	Growth
Chloramphenicol	1 μg/ml	1222	Growth
	10 μg/ml	25	No growth
	50 μg/ml	23	No growth
Erythromycin	1 μg/ml	27	No growth
	5 μg/ml	32	No growth
	50 μg/ml	28	No growth
Tetracycline	1 μg/ml	39	Growth
	5 μg/ml	31	No growth
	50 μg/ml	26	No growth

Concentration of cells, 10^{-2} dilution of an 18- to 20-hr culture. Counts/min have background and sterile controls subtracted and are averages of duplicates.

at 5 μg concentration was even more pronounced than at 1 μg. However, at 50 μg there was a constant decrease in $C^{14}O_2$ evolved, clearly demonstrating the effect of time as well as drug concentration on the reaction of the culture to the tetracycline. Again this correlated with the results of the tube-dilution determination.

The data shown in Table 4 illustrate the importance of the time of collection of the $C^{14}O_2$ if only a single determination is made. As can be seen, there was complete correlation with the results of the tube-dilution determinations with the exception of the values at 1 μg of tetracycline. On the basis of the 6-hr studies shown in Table 3 one would assume that the amount of $C^{14}O_2$ increased later at the 1 μg concentration. However if one considers the results of any of the antibiotics as a block or unit, sensitivity or lack of it can still be determined.

The problem of cell concentration versus antibiotic concentration and time of determination is present in the radioisotope method just as it is in the conventional methods. This is illustrated by the data in Table 5. It can be seen that the activity detected at 4 hr was not sufficient to determine sensitivity but that at 5 or 6 hr the trends correlated with the tube-dilution determinations. The low control values point to the problem of generally low activity and would prevent a false determination of sensitivity.

Table 5. Effects of Antibiotics on $C^{14}O_2$ Released by Achromobacter (10949) over 6 hr

Antibiotic	Concentration	Counts/min						Tube dilution, 24 hr
		1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	
None		0	2	5	3	89	174	
Penicillin	1 unit/ml	0	5	6	4	18	70	Growth
	5 units/ml	3	2	6	0	41	85	Growth
	50 units/ml	0	2	6	2	49	98	Growth
Tetracycline	1 μg/ml	0	5	30	24	29	80	Growth
	5 μg/ml	4	3	10	0	20	79	Growth
	50 μg/ml	1	5	3	4	4	10	No growth

Cell concentration, 10^{-2} dilution of 18- to 20-hr culture. Counts/min have background and sterile controls subtracted and are averages of duplicates.

DISCUSSION

The data presented are representative of a number of determinations which show that the sensitivity of some bacteria can be determined readily within 4 hr either by using a $C^{14}O_2$ collection at 2 and 4 hr and observing trends or, in some cases, with a single collection. The most reliable determinations are those in which trends are observed and it is this method which is favored by the authors. There are data which show that in some cases 1- and 3-hr determinations are satisfactory although none of these have been included in this report.

By collecting $C^{14}O_2$ early and continuing to collect at regular intervals it is possible to observe the interaction between the culture and the antibiotics. In this way one can see the effect of time and it is possible that with some variations in the method bacteriostatic or bactericidal effects of the antimicrobial agents could be determined within a relatively short period of time.

Although a number of Gram-positive and Gram-negative bacteria have been investigated there is still a need for more to be studied. There is also a need for

a greater degree of standardization of size of inoculum so that all bacteria react within 3 or 4 hr.

Investigations are being carried out presently to study the above problems and the direct application of the radioisotope method to body fluids. In this connection some studies have been done on urine specimens and, in the limited number of specimens tested, good results have been achieved in determining sensitivity directly. However, there appears to be a lack of correlation between $C^{14}O_2$ released and the numbers of bacteria present. The investigations are being continued.

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SUMMARY

The antimicrobial activity of various antibiotics is determined by using a radioisotope method. The data show that antibiotic sensitivity of bacteria isolated from patients can be determined within 4 hr and are in agreement with results obtained from tube-dilution sensitivity tests done concurrently. The application of the radioisotope method to the direct determination of sensitivity of bacteria in material freshly obtained from patients is being investigated currently.

REFERENCE

1. Levin, G. V., et al. Rapid, radioactive test for coliform organisms. J. Am. Water Works Assoc. 51: 101-104. 1959.

DISCUSSION

DR. BONDI: Dr. Heim, do I understand that potentially you could test any organism by this method? Do all organisms have this enzyme system? What is the enzyme? What is the mechanism?

DR. HEIM: We selected formate because of the fact that it is a single-carbon compound. There are many ways that organisms can do things to formate, making it open to the possibility that it would be universally used. Naturally, we haven't tested all organisms. We have done roughly 100 determinations of this sort and we have yet to find one that doesn't work, and this includes Dr. Waksman's favorites, the streptomycetes. They and all the bacteria that we have tried, both Gram-negative and Gram-positive, will do it. The rate of evolution of carbon dioxide will vary even from one staphylococcal strain to another.

DR. BONDI: What about a Gonococcus?

DR. HEIM: We haven't tried it.

DR. CARR: I wonder if I could ask some technical questions here. What kind of counter are you using?

DR. HEIM: Window counter. Nuclear-Chicago model with an M5 changer.

DR. CARR: I understand you incubate your organisms. Is the culture vessel covered while you incubate it?

DR. HEIM: We do this and run duplicates in a petri dish. We have tried covering it with large cover slips and we find that it makes very little difference for our purposes.

DR. CARR: And then you place something containing barium hydroxide on top of this culture planchet?

DR. HEIM: Yes, an inverted planchet which contains only a 25-mm-diameter paper pad soaked with barium hydroxide solution.

Q: I suspect that you are not recommending this for routine use at the present time. The techniques involved would probably go beyond the capabilities of most clinical bacteriology laboratories. But I think that this is certainly a commendable form of enterprise and I wonder if you would comment on the technical

problems involved? How difficult is it to carry out one of these procedures on a routine basis? It is apparent too that you can't use this with an organism that is growing anaerobically?

DR. HEIM: The ease or the simplicity of the test possibly is best illustrated if I tell you that we have used this sort of thing for two years now in our medical student's laboratory. We actually have the medical students carry out the reaction.

DR. SAZ: How do you explain the fact that even with low concentrates of antibiotics you are getting much higher counts in the media than you are in the controls?

DR. HEIM: We think this may depend on the size of the inoculum. As I am sure you realize, this is an extremely sensitive method of detection.

Q: Let's assume that the antibiotics are inhibiting the growth of the culture. Therefore one would assume that any acid which is produced would be minimal under such a situation. Now, in the control without antibiotics the organisms are growing and conceivably are producing acid. In such a circumstance might you not lose CO_2 to the atmosphere because of the lower pH in the controls? In the antibiotic-containing planchets you would not be losing CO_2 to the atmosphere as much because the pH is not decreasing. Therefore, you might expect under these circumstances to have a higher count, not that they actually produce more but because they have lost less from the culture medium.

DR. HEIM: Except that you might also have this evolution going on while you are collecting the labeled CO_2.

Q: Except for very fastidious organisms a tube-dilution test or a disc test can give you sensitivity information on many organisms in, let's say, 6 hr. What does this simple sensitivity test do that a conventional disc test does not do?

DR. HEIM: The way it is set up now, nothing. We think it has potential as a laboratory tool to study trends or interactions. It doesn't tell you any more but shortens the period of time in some cases. I don't want to become involved in a definition of the value of bacteriostatic versus bactericidal, but there is the possibility that this can be shown within a few hours by this method.

Q: Don't you think it's important that in four hours you can tell whether or not an antibiotic is effective on the organism?

DR. HEIM: In some cases, yes. In other cases the time that you start an antibiotic isn't lifesaving. If a hospital or medical center had facilities for doing these tests quickly, clinicians who normally do not use sensitivity tests might be willing to do so if they could have results by the end of the day.