Metabolic Inhibition as an Index of Bacterial Susceptibility to Drugs

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Detection of bacterial growth by measuring the release of 14CO2 from the metabolism of uniformly labeled 14C-glucose has proven to be accurate, sensitive, and rapid. The inhibition of glucose metabolism by antibiotics as an index of bacterial susceptibility was evaluated based on radiometric methodology. Bacterial dose-response curves to antibiotics were defined. The susceptibility to drugs was determined within several hours after inoculation of the cultures. The pentose phosphate shunt appeared to be the major metabolic pathway involved during this period. There was a consistent relationship between the dose-response curves determined by the inhibition of glucose metabolism and the minimal inhibitory concentrations determined by a serial broth dilution technique. Inhibition of metabolism as an index of bacterial susceptibility to drugs appears to be valid, rapid, and readily applicable to available automation.

In 1966, a report from the National Institutes of Health (2) stated that a universally accepted or standardized method for testing bacterial susceptibility to drugs had not been developed. A 1970 report from the Center for Disease Control expressed the view that no one in vitro antimicrobial susceptibility test satisfactorily provided the clinician with useful laboratory information which would be helpful in selecting the therapy for all bacterial diseases (10). In recognition of these problems, the World Health Organization sponsored a series of international study groups (7, 11). One of the four recommendations from the 1971 report (7) stressed "the need for ... the development and evaluation of new techniques for measuring sensitivity."

The tests most frequently employed to measure bacterial susceptibility to drugs in clinical laboratories are agar diffusion, agar dilution, and broth dilution. Many variables can affect the results of these procedures, not the least of which is visual interpretation of results. A radiometric method for the detection of bacterial growth, based on bacterial metabolism of glucose, has been developed (4-6) which reduces variables to a minimum and, of greater significance, offers objective evidence of bacterial growth. The purpose of this investigation was to evaluate the inhibition of bacterial metabolism of glucose as an index of bacterial susceptibility to drugs.

MATERIALS AND METHODS

Eight bacterial species were evaluated in this study. With the exception of Escherichia coli and Staphylococcus aureus (both of which are American Type Culture Collection cultures), all bacterial strains were originally recovered from clinical specimens submitted to the clinical microbiology laboratory of the University of Florida Teaching Hospital and maintained as stock cultures. The microorganisms studied were E. coli ATCC 25922, S. aureus ATCC 25923, Proteus mirabilis 2, Enterobacter 3, enterococcal strain 2, Pseudomonas 10, alpha-hemolytic Streptococcus G76, and Aerobacter aerogenes 7.

The stock cultures were maintained on Trypticase soy agar slants under refrigeration at 4°C and were transferred to fresh slants every 2 weeks. Prior to each investigation, the selected microorganism was transferred to 10 mL of Trypticase soy broth (BBL) from the stock culture and incubated for 18 hr at 37°C. By using cultures that had been incubated for 18 hr, it was possible to obtain bacterial concentrations of 10⁴ to 10⁶ organisms by means of standard dilutions. The bacterial concentration in 18-hr cultures was determined concurrently by inoculating blood agar plates (BBL) with 0.1 mL of the diluted bacterial suspension. The inoculum was evenly spread over the agar surface of the plate with a sterile bent rod and, after overnight incubation at 37°C, colony counts were determined. Colony counts for each specimen were determined in triplicate.

Each bacterial species was evaluated against the following 10 antibiotics: chloramphenicol, erythromycin, kanamycin, streptomycin, gentamicin, penicillin G,
ampicillin, cephalothin, colistin, and tetracycline. Antibiotic solutions were freshly prepared for each test. The antibiotics were reconstituted in sterile, distilled, demineralized water, and the required dilutions were made with Trypticase soy broth just prior to use.

**Broth dilution method.** Serial dilutions of the antibiotics were made in 0.5 ml of Trypticase soy broth in tubes 13 by 100 mm. To each dilution, and a control, 0.5 ml of a diluted 18 hr pure bacterial culture was added for a final volume of 1.0 ml. Each tube contained approximately 5 x 10^8 organisms per ml. After 18 hr of incubation at 37 C, the tubes were inspected for bacterial growth. The minimal inhibitory concentration (MIC) of the antibiotic corresponded to the lowest concentration of drug without visible turbidity or sedimentation. The bactericidal concentration of the antibiotic was determined as the greatest dilution at which no organisms grew from subcultures of the tubes.

**Metabolic inhibition method.** The principle of the metabolic inhibition method of determining bacterial susceptibility to drugs was based on the changes detected in the release of \(^{14}\)CO\(_2\) from the metabolism of uniformly labeled \(^{14}\)C-glucose.

The technique for measuring \(^{14}\)CO\(_2\) from \(^{14}\)C-glucose has been previously described (4), and only an abbreviated description will be given here as it applies to this investigation. The instrument (Bactec R, Johnston Laboratories) used in this study is capable of handling 25 culture vials, periodically sampling the atmosphere within the vials for the presence of \(^{14}\)CO\(_2\), and quantitating the amount of \(^{14}\)C detected. The level of radioactivity measured by the ion chamber is expressed as "Bactec index of CO\(_2\)." By means of known standards of \(^{14}\)CO\(_2\), 1 "Bactec index unit" was determined to be the equivalent of \(5.8 \times 10^{-4}\) \(\mu\)Ci of carbon for the instrument used in this investigation.

The 50-ml culture vials contained 10 ml of glucose-free Trypticase soy broth (BBL) and 1.0 \(\mu\)Ci of uniformly labeled \(^{14}\)C-glucose. Samples of serially diluted antibiotics were added to the vials to give a final antibiotic concentration comparable to that used for the serial broth dilution method. To obtain a final concentration in the culture vials of approximately \(5 \times 10^8\) microorganisms, 1-ml inocula of \(5 \times 10^8\) organisms were added to each vial. Two control cultures were used for each study; one with bacteria but without antibiotics, to measure maximal uninhibited \(^{14}\)C-glucose metabolism, and the other without bacteria or antibiotics, to measure background radioactivity. After first the antibiotics and then the bacterial inoculum had been added to the culture vials, the vials were immediately placed in the detecting instrument which was programmed to sample each culture vial hourly, measure the \(^{14}\)CO\(_2\) from the atmosphere of each vial, and record the results as an index of metabolism. Only the incubation period from the time of inoculation to the maximal utilization rate of \(^{14}\)C-glucose by the control culture was used for the determination of the inhibitory effect of antibiotics on the release of \(^{14}\)CO\(_2\) from the metabolism of \(^{14}\)C-glucose. The quantity of \(^{14}\)CO\(_2\) released by the culture vials containing antibiotics was expressed as a percentage of the control culture.

**RESULTS**

In this system, approximately 1.0 \(\mu\)g of uniformly labeled \(^{14}\)C-glucose is used as the sole source of glucose. With this extremely limited quantity of glucose, the \(^{14}\)CO\(_2\) was released as a bimodal curve with *E. coli*, Enterobacter, and *A. aerogenes*. Figure 1 illustrates the bimodal release of \(^{14}\)CO\(_2\) from glucose metabolism by an inoculum of \(5 \times 10^5\) cells of *E. coli*. A primary peak of activity was observed after 3 hr of incubation and a secondary peak occurred after 7 hr. The other organisms demonstrated a unimodal curve of \(^{14}\)CO\(_2\) release; Fig. 2 illustrates the release of \(^{14}\)CO\(_2\) by the metabolic activity of *S. aureus*. After 4 hr of incubation, a single peak was observed.

Since one metabolic pathway would be more desirable for quantitation of metabolic inhibition, a limited investigation of the bimodal release of \(^{14}\)CO\(_2\) was undertaken. Explanations for this phenomenon could be (i) a bacterial contaminant, (ii) heterogeneity of the culture, (iii) two distinct periods of rapid growth, (iv) a rapid decrease in pH of the culture media, and (v) multiple metabolic pathways.
Bacterial contamination was ruled out by the growth of homogeneous colonies on blood agar culture plates after several inoculations from the original culture vial.

To exclude the possibility of a heterogeneous organism, the E. coli culture was cloned successively four times, and a sample of the Trypticase soy broth suspension from the last clone was inoculated into a Bactec vial, incubated, and sampled hourly. After the cloning procedure, the time-rate release of $^{14}$CO$_2$ was bimodal and essentially identical to the results from the original culture.

Huntington and Winslow (9) demonstrated that the greatest CO$_2$ production by bacterial metabolism occurs during the period of exponential growth. If a bacterial species should manifest two distinct periods of rapid growth, a bimodal release of $^{14}$CO$_2$ could occur. Figure 1 demonstrates that the first peak release of $^{14}$CO$_2$ by E. coli occurred during the exponential growth period, whereas the second peak occurred at the beginning of the stationary phase. These findings are not suggestive of two distinct periods of bacterial growth.

The solubility of CO$_2$ in an aqueous solution decreases with decreasing pH, and a rapidly increasing acidity of the culture media might account for the secondary increase of $^{14}$CO$_2$ in the bimodal curves. However, hourly pH measurements of the culture media demonstrated no change during the first 6 hr of incubation followed by a slowly increasing pH to 8.25 by the 17th hr.

To determine whether more than one metabolic pathway was involved in the release of $^{14}$CO$_2$, all microorganisms in this study were evaluated with glucose labeled at the carbon-1 position and at the carbon-6 position. Bimodal organisms (e.g., E. coli) produced $^{14}$CO$_2$ from $^{14}$C-1-glucose predominantly during the first peak and from $^{14}$C-6-glucose during the second peak (Fig. 3). The metabolism of $^{14}$C-1-glucose by the unimodal organisms (e.g., S. aureus) was similar to that of uniformly labeled $^{14}$C-glucose, whereas there was little production of $^{14}$C-6-glucose (Fig. 6). These results suggested that with certain organisms more than one metabolic pathway was involved. Because of certain physical characteristics of the radiometric system (to be discussed later), the first and second periods of $^{14}$CO$_2$ release for bimodal organisms overlap. Nevertheless, changes in metabolism up to the first peak would be useful information if this point occurred before the onset of the second metabolic function. With 10-min sampling for $^{14}$CO$_2$ of a culture of E. coli, it was observed that the first metabolic function is almost completely distinct from the second function and that the onset of the second function occurs after the peak of the first function (Fig. 4). Calculations of the quantity of $^{14}$C detected during the first phase of the bimodal response (based on 10-min sampling) were equivalent to 16 to 17% of the total $^{14}$C present in the $^{14}$C-glucose substrate. Since 16 to 17% is equal to one-sixth of the glucose carbons, it follows that this probably represents a single carbon position. These findings combined with those from $^{14}$C-1-glucose and $^{14}$C-6-glucose studies suggested that the first metabolic pathway in this system was probably the monophosphate shunt only (1). Based on these findings, measurement of antibiotic inhibition of glucose metabolism was limited to the first portion of the metabolic cycle.

In this automated system the total production of $^{14}$CO$_2$ is never measured because the solubility of CO$_2$ is 100% at the pH of the culture (7.2). To measure the relationship of the detected $^{14}$CO$_2$ to the actual amount produced, 10 culture vials (with 1 μCi of uniformly labeled $^{14}$C-glucose added and inoculated with E. coli) were sampled hourly for the release of $^{14}$CO$_2$. At each hour hydrochloric acid was added to one vial, which reduced the pH to less than 1 and the CO$_2$ solubility to nearly zero, and the culture was immediately resampled to measure the residual free $^{14}$CO$_2$. The amount of $^{14}$CO$_2$ sampled ranged from 34 to 39%
Fig. 3. Release of $^{14}$CO$_2$ from metabolism of $^{14}$C-glucose versus bacterial growth by Escherichia coli. The primary peak release of $^{14}$CO$_2$ occurs during exponential growth, and the second peak is at the beginning of the stationary phase of bacterial growth.

of the total $^{14}$CO$_2$ in both the atmosphere and the culture media (Fig. 5) during the first 5 hr of culturing. Since the fraction of released $^{14}$CO$_2$ measured was relatively constant, a control culture could be used as a reference for measuring the metabolic inhibition due to antibiotics.

The quantity of $^{14}$C-glucose available for bacterial metabolism is very limited. The first peak release of $^{14}$CO$_2$ indicates that the maximal rate of metabolism has been achieved for that particular pathway, and this point occurs during the exponential phase of bacterial growth (Fig. 1). After the peak $^{14}$CO$_2$ release, the decreasing rate of $^{14}$CO$_2$ production (still during exponential bacterial growth) suggests that the available glucose is now inadequate for an optimal rate of metabolism and that this period of incubation cannot be used for comparison purposes. Therefore, metabolic inhibition was measured only up to the peak release of $^{14}$CO$_2$.

To evaluate the relationship between metabolic inhibition and the selectivity of action of the antibiotics, the drugs were grouped according to their mode of action. Figure 6 illustrates the metabolic inhibition curves for six antibiotics that interfere with genetic function. For E. coli, the inhibitory concentrations by the broth dilution technique correlated with the radiometric method for drug concentrations that inhibited $^{14}$CO$_2$ in the range of 10 to 30%. For those antibiotics that affect the cell wall, the MIC values correlated with drug concentrations that showed little inhibition of glucose metabolism (Fig. 7). These findings for E. coli were typical of all of the bacterial studied, although there were occasional exceptions.

All of the microorganisms and antibiotics evaluated in this study demonstrated a progressive in-
DRUG INHIBITION OF BACTERIAL METABOLISM

Fig. 4. Comparison of $^{14}$CO$_2$ detected by 10-min and 1-hr sampling of Escherichia coli cultures. The 10-min sampling of the culture vials demonstrates that the bimodal release of $^{14}$CO$_2$ from uniformly labeled $^{14}$C-glucose is two distinct metabolic cycles. The demarcation between the two peaks of $^{14}$CO$_2$ is nearly complete.

Fig. 5. Comparison of $^{14}$CO$_2$ in culture media and air with Escherichia coli cultures. The distribution of $^{14}$CO$_2$ between the atmosphere and the culture media is relatively constant during the lag and exponential growth phase of E. coli.

inhibition of metabolism which ranged from no effect to nearly complete inhibition, and the diminishing metabolic rate extended over a range of five to seven drug concentrations. In no instance was metabolic inhibition precipitous, i.e., from no inhibition to marked or complete inhibition over a range of only one or two drug concentrations.

DISCUSSION

The 10 antibiotics studied in this investigation fall into three general classifications according to their modes of action: (i) interference with protein synthesis and genetic function, (ii) interference with cell wall structure and function, and (iii) interference with membrane function (8). These drugs do not appear to affect directly the metabolism of glucose, but "the biochemical processes of bacterial cells are closely interlinked. Hence, disturbance of any one important system is likely to have effects on many others" (8). The tetracyclines have been shown to limit glucose oxidation and respiration in bacteria, but there is little evidence indicating that the primary site of antibiotic action is the glucose oxidation or respiratory pathways (3).

When inhibition of glucose metabolism and the MIC of antibiotics affecting protein synthesis and genetic function are compared (Fig. 6), the concentration of drugs required for a minimal
Antibiotic concentration in μg or Units

FIG. 6. Inhibition of glucose metabolism versus minimal inhibitory concentration (MIC) for antibiotics affecting protein synthesis. The test organism was Escherichia coli. The arrows indicate the MIC by serial broth dilution for each antibiotic.

FIG. 7. Inhibition of glucose metabolism versus minimal inhibitory concentration (MIC) for antibiotics affecting cell walls. The test organism was Escherichia coli. The arrows indicate the MIC by serial broth dilution for each antibiotic.

inhibitory effect (serial broth dilution) produced marked inhibition of glucose metabolism. Figure 8 illustrates the relationship between E. coli bacterial generation and glucose metabolism for several concentrations of tetracycline. At a concentration of 0.8 μg/ml, bacterial generation was retarded but not inhibited. The area under the ¹⁴CO₂ curve up to 4 hr of incubation (peak release of ¹⁴CO₂ for control) was less than half that of the control. At the MIC of tetracycline (1.6 μg/ml), the release of ¹⁴CO₂ was only 38% of control. These findings suggest that the meta-
bolic systems affected by this group of drugs are particularly sensitive.

For drugs affecting the cell wall (Fig. 7), greater concentrations of antibiotics were required to inhibit glucose metabolism than to produce a minimal inhibitory effect by the broth dilution technique. Approximately 9 μg of ampicillin per ml was required to reduce the release of 14CO2 to 50% of the control, whereas the MIC was found to be 1.6 μg/ml. These findings suggest that greater concentrations of drugs are required to affect glucose metabolism than to affect the cell wall.

In this study, the serial broth dilution technique
Table 1. Bacterial susceptibility to antibiotics by serial broth dilution versus inhibition of glucose metabolism

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (μg/mL)</th>
<th>uCO₂ (μg/mL)</th>
<th>uCO₂/MIC</th>
<th>MIC (μg/mL)</th>
<th>uCO₂ (μg/mL)</th>
<th>uCO₂/MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol</td>
<td>5.6</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>4.0</td>
<td>3.0</td>
<td>0.75</td>
<td>4.0</td>
<td>3.0</td>
<td>0.75</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>1.0</td>
<td>1.5</td>
<td>1.5</td>
<td>1.0</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>3.5</td>
<td>3.0</td>
<td>0.85</td>
<td>3.5</td>
<td>3.0</td>
<td>0.85</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>6.0</td>
<td>6.0</td>
<td>1.0</td>
<td>6.0</td>
<td>6.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>0.4</td>
<td>0.5</td>
<td>1.25</td>
<td>0.4</td>
<td>0.5</td>
<td>1.25</td>
</tr>
<tr>
<td>Colistin</td>
<td>0.1</td>
<td>0.2</td>
<td>2.0</td>
<td>0.1</td>
<td>0.2</td>
<td>2.0</td>
</tr>
</tbody>
</table>

The minimal inhibitory concentration (MIC) is the lowest concentration of antibiotic (in micrograms) by the uCO₂ method that concentration (in micrograms) required to attain a 50% reduction was used as a comparison reference for the ¹⁴CO₂ radiometric method. The validity of this comparison is certainly questionable, because the incubation period was several hours for the ¹⁴CO₂ method and 18 hr for broth dilution, and because one method measures the inhibition of glucose metabolism and the other measures the inhibition of bacterial generation. Nevertheless, the results of the ¹⁴CO₂ method do demonstrate a consistency relative to the broth dilution technique and also demonstrate that inhibition of glucose metabolism can be used as an index of bacterial susceptibility to antibiotics. Table 1 compares the MIC of the antibiotics found by the broth dilution technique and the concentration of antibiotics required to attain a 50% reduction in glucose metabolism.

The radiometric technique offers quantitative and objective results for measuring bacterial susceptibility to drugs. In addition, the relatively short incubation period required for determining bacterial susceptibility is of primary clinical importance.

ACKNOWLEDGMENTS

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LITERATURE CITED

ERRATUM

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Volume 2, no. 5, p. 405: change author by-line to read as follows “FRANK H. DELAND AND MICHAEL COHEN, Division of Nuclear Medicine, Veterans Administration Hospital, and University of Florida, Gainesville, Florida 32601.”

On page 412 add to Acknowledgments: “A portion of this study was supported by Public Health Service grant 5TIAI from the National Institute of Allergy and Infectious Diseases and represents a portion of the thesis of M.C. submitted to the Graduate School, University of Florida, Gainesville, Fla. 32601.”

ANTIMICROBIAL AGENTS
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CHEMOTHERAPY

A. Hada