

Antibiotics in Laboratory Medicine

VICTOR LORIAN, M.D.
Editor

Antibiotics in Laboratory Medicine

VICTOR LORIAN, M.D.
Editor

Chairman, Division of Microbiology and Epidemiology
Department of Pathology,
The Bronx-Lebanon Hospital Center, Bronx, New York
Professor of Laboratory Medicine
Albert Einstein College of Medicine
Bronx, New York



WILLIAMS & WILKINS
Baltimore/London

Copyright ©, 1980
The Williams & Wilkins Company
428 E. Preston Street
Baltimore, Md 21202, U.S.A.

All rights reserved. This book is protected by copyright. No part of this book may be reproduced in any form or by any means, including photocopying, or utilized by any information storage and retrieval system without written permission from the copyright owner.

Made in the United States of America

Library of Congress Cataloging in Publication Data

Main entry under title:

Antibiotics in laboratory medicine.

Includes index.

1. Microbial sensitivity tests. I. Lorian, Victor. [DNLM: 1. Microbial sensitivity tests. 2. Antibiotics—Pharmacodynamics. QW25.3 A629]

QR69.A57A57 615'.329 79-9822

ISBN 0-683-05166-0

Cover Illustration: A staphylococcus bursting under the effect of an antibiotic.

Composed and printed at the
Waverly Press, Inc.
Mt. Royal and Guilford Aves.
Baltimore, Md 20202, U.S.A.

Preface

Antibiotics* are among the most valuable and the most utilized therapeutic agents in medical practice. They account for over 30% of the budgets of hospital pharmacies. *In vitro* tests are the main guidelines for monitoring antibacterial therapy.

The growth of the field of antibiotic research and therapy, as related to the clinical laboratory, has resulted in an extensive literature. It is the purpose of *Antibiotics in Laboratory Medicine* to present in one reference volume the fundamental *in vitro* aspects of antibiotics, from the screening for new agents to their assay in various body fluids.

I have compiled this volume together with experts who have pioneered and developed by their own investigations the various areas of research and practice with antibiotics. Our efforts culminated in this comprehensive reference book. It contains an unprecedented amount of theoretical and practical information as to what can be done in the laboratory including methodology and the significance of results.

Each chapter contains an academic section which covers the field at the highest level of current expertise, as well as a practical part which describes in detail not only sophisticated technology, but also routine methods in such a way that scientists, physicians, and technologists should be able to reproduce the procedures described without the need for additional reading. Consistent with this approach, some duplication was not only accepted but also required.

* In this volume the word **antibiotic** will be used to cover all antimicrobial agents.

The reader can find all the specific information in the chapter consulted without the need of reference to other parts of the book. Each chapter is documented by an extensive bibliography.

It is my hope that this volume will help those in research as well as those whose daily task is concerned with the many laboratory aspects required for the care of patients receiving antibiotics.

It is an honor to introduce this book containing contributions from such eminent professionals who have donated so much of their valuable time and expertise toward its preparation. Their cooperation made the task of editing a real pleasure. I am confident the results of their efforts will prove to be invaluable to all those concerned in this field.

Special recognition must go to Drs. Max Finland, L. D. Sabath, and John Sherris who enthusiastically helped me to undertake the publication of this volume. My gratitude also goes to my Chief and Friend, Dr. Leopold Reiner, and to the administration of The Bronx Lebanon Hospital Center for creating conditions that allowed me to concentrate on the many endeavors required for the editing of this volume. Last but not least my sincere thanks to Mrs. Urbis C. Westray, my secretary, whose devoted help made this complex activity possible.

I would also like to thank all my friends at Williams & Wilkins whose expertise and kind cooperation made the process of publishing an enjoyable experience.

VICTOR LORIAN

New York, 1980

Contributors

Jacques F. Acar, M.D.

Professor of Medical Microbiology, Université Pierre et Marie Curie, Hôpital St. Joseph, Institut Pasteur, Paris, France

Barbara Atkinson, M.A., MT (ASCP)

The Bronx Lebanon Hospital Center, Bronx, New York

Albert Balows, Ph.D.

Director, Bacteriology Division, Bureau of Laboratories, Center for Disease Control, Atlanta, Georgia

Arthur L. Barry, Ph.D.

Director, Microbiology Laboratories, University of California, Davis, Medical Center, Sacramento, California and Associate Professor in Clinical Microbiology, University of California at Davis, School of Medicine, Davis, California

David M. Carlberg, Ph.D.,

Professor, Department of Microbiology, California State University, Long Beach, California

Herman Chmel, M.D.

Assistant Professor of Medicine, Department of Medicine and Preventive Medicine, New Jersey College of Medicine and Dentistry, Newark; and Infectious Disease Section, Medical Service, Veterans Administration Hospital, East Orange, New Jersey

Roy Cleeland, Ph.D.

Director, Department of Chemotherapy, Hoffmann-La Roche Inc., Nutley, New Jersey

William A. Craig, M.D.

Associate Professor of Medicine, University of Wisconsin-Madison, Chief, Infectious Diseases, William S. Middleton Memorial Veterans Administration Hospital, Madison, Wisconsin

Julian Davies, Ph.D.

Professor of Biochemistry, Department of Biochemistry, University of Wisconsin-Madison, Madison, Wisconsin

Stephen C. Edberg, Ph.D.

Associate Professor of Pathology, Albert Einstein College of Medicine, Bronx, New York and Division of Microbiology and Immunology, Department of Pathology, Montefiore Hospital and Medical Center, Bronx, New York

Josephine M. Ehret, B.S., MT (ASCP)

Research Microbiologist, Department of Medicine, Division of Infectious Diseases, University of Colorado Medical Center, Denver, Colorado

Theodore C. Eickhoff, M.D.

Professor and Head, Division of Infectious Disease, University of Colorado, Health Sciences Center, Denver, Colorado

Lynn P. Elwell, Ph.D.

Burroughs Wellcome Company, Research Triangle Park, North Carolina

Denis A. Evans, M.D.

Channing Laboratory, Harvard Medical School and Department of Medicine, Peter Bent Brigham Hospital, Boston, Massachusetts

Stanley Falkow, Ph.D.

Professor of Microbiology and Medicine, University of Washington, School of Medicine, Seattle, Washington

Thomas L. Gavan, M.D.

Chairman, Department of Microbiology, The Cleveland Clinic Foundation, Cleveland, Ohio

Emanuel Grunberg, Ph.D.

Director, Chemotherapy and Diagnostics, Hoffmann-La Roche Inc., Nutley, New Jersey

Edward H. Kass, M.D., Ph.D.

William Ellery Channing Professor of Medicine, Harvard Medical School, Director, Channing Laboratory, Peter Bent Brigham Hospital, Boston, Massachusetts

Donald J. Krogstad, M.D.

Microbiology Laboratory, Divisions of Laboratory Medicine and Infectious Disease, Departments of Medicine and Pathology, Barnes Hospital, Washington University School of Medicine, St. Louis, Missouri

Victor Lorian, M.D.

Chairman, Division of Microbiology and Epidemiology, Department of Pathology, The Bronx Lebanon Hospital Center, Bronx, New York; Professor of Laboratory Medicine, Albert Einstein College of Medicine, New York, New York

Donald B. Louria, M.D.

Professor and Chairman, Department of Preventive Medicine and Community Health, New Jersey Medical School, Newark, New Jersey

J. Kenneth McClatchy, Ph.D.

Chief of Clinical Laboratories, National Jewish Hospital and Research Center, Denver, Colorado

Barbara H. Minshew, Ph.D.

Associate Professor, Department of Surgery, University of Washington, School of Medicine, Seattle, Washington

Robert C. Moellering, Jr., M.D.

Associate Professor of Medicine, Harvard Medical School; Associate Physician, Infectious Disease Unit, Massachusetts General Hospital, Boston, Massachusetts

Harold C. Neu, M.D.

Professor of Medicine and Pharmacology, Head, Division of Infectious Diseases, College of Physicians and Surgeons, Columbia University, New York, New York

Robert A. Rippere, B.S.

Microbiology Assay Branch, National Center for Antibiotics Analysis, Food and Drug Administration, Washington, D.C.

Jon E. Rosenblatt, M.D.

Consultant in Clinical Microbiology and Infectious Diseases, Mayo Clinic, Rochester, Minnesota

L. D. Sabath, M.D.

Professor of Medicine, Head, Section on Infectious Diseases, University of Minnesota, School of Medicine, Minneapolis, Minnesota

Pravinkumar B. Sehgal, M.B.B.S., Ph.D.

Assistant Professor, The Rockefeller University, New York, New York

John C. Sherris, M.D.

Professor and Chairman, Department of Microbiology and Immunology, University of Washington, Seattle, Washington

Byungse Suh, M.D., Ph.D.

Assistant Professor of Medicine and Microbiology, Section of Infectious Diseases, Temple University Health Sciences Center, Philadelphia, Pennsylvania

Igor Tamm, M.D.

Professor and Senior Physician, The Rockefeller University, New York, New York

Clyde Thornsberry, Ph.D.

Chief, Antimicrobics Investigations Section, Center for Disease Control, Atlanta, Georgia

Lauri D. Thrupp, M.D.

Professor of Medicine, Infectious Disease Division, University of California, Irvine; Consultant, Clinical Microbiology Section, Laboratory Service, and Chief, Infection Control, Infectious Disease Service, University of California Irvine Medical Center, Irvine, California

Contents

	Preface	v
	Contributors	vii
	List of Tables	xi
Chapter 1	Procedures for Testing Antibiotics in Agar Media: Theoretical Considerations ARTHUR L. BARRY, PH.D.	1
Chapter 2	The Disc Susceptibility Test JACQUES F. ACAR, M.D.	24
Chapter 3	Principles of Optical and Electrical Methods for the Determination of Bacterial populations DAVID M. CARLBERG, PH.D.	55
Chapter 4	Susceptibility Testing of Antibiotics in Liquid Media LAURI D. THRUPP, M.D.	73
Chapter 5	Antibiotic Susceptibility Testing for Anaerobes JON E. ROSENBLATT, M.D.	114
Chapter 6	Antituberculosis Drugs: Mechanisms of Action, Resistance, Susceptibility Testing and Assays of Activity in Biological Fluids J. KENNETH MCCLATCHY, PH.D.	135
Chapter 7	Antifungal Antibiotics: Mechanism of Action, Resistance, Susceptibility Testing and Assays of Activity in Biological Fluids HERMAN CHMEL, M.D., AND DONALD B. LOURIA, M.D.	170
Chapter 8	Automation in Antibiotic Susceptibility Testing CLYDE THORNSBERRY, PH.D.	193
Chapter 9	Determination of Antibiotic Levels in Body Fluids: Techniques and Significance. Bactericidal Tests in Endocarditis and Other Severe Infections STEPHEN C. EDBERG, PH.D., AND LEON D. SABATH, M.D.	206
Chapter 10	Protein Binding and the Antibacterial Effects. Methods for the Determination of Protein Binding WILLIAM A. CRAIG, M.D. AND BYUNGSE SUH, M.D., PH.D.	265

Chapter 11	Combinations of Antibiotics, Mechanisms of Interaction Against Bacteria	298
	DONALD J. KROGSTAD, M.D., AND ROBERT C. MOELLERING, JR., M.D.	
Chapter 12	Effects of Subminimum Inhibitory Concentrations of Antibiotics on Bacteria	342
	VICTOR LORIAN, M.D.	
Chapter 13	Quality Control Methods for In Vitro Antibiotic Susceptibility Testing	409
	ALBERT BALOWS, PH.D., AND THOMAS L. GAVAN, M.D.	
Chapter 14	Mutational Antibiotic Resistance	418
	JOHN C. SHERRIS, M.D., AND BARBARA H. MINSHEW, PH.D.	
Chapter 15	The Characterization of Plasmids that Carry Antibiotic Resistance Genes	433
	LYNN P. ELWELL, PH.D., AND STANLEY FALKOW, PH.D.	
Chapter 16	Antibiotic Inactivating Enzymes and Bacterial Resistance	454
	HAROLD C. NEU, M.D.	
Chapter 17	Aminoglycoside-Aminocyclitol Antibiotics and Their Modifying Enzymes	474
	JULIAN DAVIES, PH.D.	
Chapter 18	The Use of Antibacterial Agents as Tools in Epidemiology and Taxonomy	490
	THEODORE C. EICKHOFF, M.D., AND JOSEPHINE M. EHRET, B.S.	
Chapter 19	Laboratory Evaluation of New Antibiotics In Vitro and in Experimental Animal Infections	506
	ROY CLEELAND, PH.D., AND EMANUEL GRUNBERG, PH.D.	
Chapter 20	Preparation and Control of Antibiotic Susceptibility Discs and Other Devices Containing Antibiotics	549
	ROBERT A. RIPPERS, B.S.	
Chapter 21	Antiviral Agents: Determination of Activity	573
	PRAVINKUMAR B. SEHGAL, M.B.B.S., PH.D., AND IGOR TAMM, M.D.	
Chapter 22	The Measurement and Significance of Antibiotic Activity in the Urine	592
	DENIS A. EVANS, M.D., AND EDWARD H. KASS, M.D.	
Chapter 23	Species Incidence, Trends of Susceptibility to Antibiotics in the U.S., and Minimum Inhibitory Concentrations	607
	BARBARA A. ATKINSON, M.A., M.T. (A.S.C.P.)	
	Index	723

List of Tables

Table 1.1. Mean Zone Sizes on Mueller-Hinton Agars from Three Commerical Sources and on a Synthetic Amino Acid Medium (SAAM) Solidified with 0.9% Lonagar and with a Synthetic Hydrogel (Neutra-Gel), all Inoculated by a Flooding Technique on Six Separate Drugs	3
Table 1.2. Precision of Diffusion Tests on Mueller-Hinton Agar (MHA) and on a Synthetic Amino Acid Medium (SAAM) Solidified with a Synthetic Polymer (Neutra-Gel), Expressed as Standard Deviations Calculated from 16 Replicate Tests	4
Table 1.3. Divalent Cation Content of 14 Different Lots of Mueller-Hinton Agar from Three Manufacturers (4-5 Lots from Each)	4
Table 1.4. CFU/ml Achieved with Three Methods for Standardizing the Inoculum for Antimicrobial Susceptibility Tests	8
Table 1.5. Carbenicillin Agar Diffusion m' Values and Agar Dilution MICs Determined as Part of a Multicenter Collaborative Study	13
Table 1.6. Effect of Anaerobiosis (Gas-Pak Jars) on Ampicillin Zone Diameters; m' vs. MIC Values with the "Seattle" Strain of <i>Staphylococcus aureus</i>	13
Table 1.7. Estimation of the Critical Time (T_0) for the Position of the Zone of Inhibition to be Determined on Mueller-Hinton Agar (MHA) and on a Synthetic Amino Acid Medium (SAAM), 10- μ g Streptomycin Discs	15
Table 1.8. Effect of Altering Disc Potency on Results of Antimicrobial Susceptibility Tests (Agar Overlay Method) with Control Strains of <i>Escherichia coli</i> (ATCC 25922) and <i>Staphylococcus aureus</i> (ATCC 25923)	20
Table 2.1. Zone-Diameter Interpretive Standards and Approximate MIC Correlates	26
Table 2.2. Zone Size Interpretive Chart	28
Table 2.3. Suggested Battery of Antibiotics for Susceptibility Testing	30
Table 2.3. Substances Which Can Be Used to Supplement Agar (MHA) and Which Do Not Interfere with the Antibiotic in the Discs	50
Table 4.1. Antimicrobial Agents for Standard Antibiotic Susceptibility Testing by Broth Dilution Tests Against Rapid Growing Pathogens in the Clinical Laboratory	76
Table 4.2. Standard Dilution Ranges of Antimicrobial Agents for Antimicrobial Susceptibility Testing in the Clinical Laboratory	78
Table 4.3. Solvents and Diluents for Stock Solutions of Antimicrobials	80
Table 4.4. Summary of Differences between Microdilution (MD) and Tube Dilution (TD) as a Function of the Growth Medium	82
Table 4.5. Susceptibility of 58 Selected Bacterial Strains to Cephalixin by Five Methods	82
Table 4.6. Effect of Growth Media on MIC (<i>Escherichia coli</i>)	83
Table 4.7. Effect of pH on Antimicrobial Activity of Antimicrobial Agents	83
Table 4.8. Increase of Ampicillin and Cephalothin MIC for 18 Bacterial Strains between 12 and 24 Hours of Incubation	85
Table 4.9. Growth of <i>Pseudomonas aeruginosa</i> 41501 in Nutrient Broth Containing Serial Dilutions of Gentamicin and Various Concentrations of Salts	86

Table 4.10. Total Concentrations of Magnesium and Calcium in Normal Serum and Commercial Batches of Mueller-Hinton (MH) Media	86
Table 4.11. Comparison of Mecillinam MIC in Different Media	87
Table 4.12. Effect of the Inoculum Concentration of <i>Escherichia coli</i> on MIC	91
Table 4.13. Effect of the Inoculum Size of <i>Pseudomonas</i> on the MIC of Carbenicillin	91
Table 4.14. Effect of the Inoculum Size on Mecillinam Activity	91
Table 4.15. Effect of Inoculum and Medium Conductivity on Mecillinam MICs	92
Table 4.16. -Fold Relationship of Broth Dilution to Agar Dilution Results	93
Table 4.17. Distribution of Broth and Agar MICs by Antibiotic—12 Laboratories Performed Both Tests Against Three Specified Reference Strains	94
Table 4.18. Relationship of Broth to Agar Dilution MICs Obtained by 12 Laboratories That Did Both Tests Against Three Specified Reference Strains	94
Table 4.19. Comparison of Microdilution and Macrodilution Results: Frequency of Agreement and Variation of Microdilution MICs when Compared with Macrodilution MICs as the Reference Standard	94
Table 4.20. Occurrence of Discrepant Test Results against <i>Pseudomonas</i> Strains, Based on the Susceptibility Test Method Used	95
Table 4.21. Reproducibility of Manual and Automated Microdilution Susceptibility Tests on Quality Control <i>Escherichia coli</i> and <i>Staphylococcus aureus</i> Strains	95
Table 4.22. A System for Preparing Dilutions for the Broth Dilution Method	96
Table 4.23. An Alternative Dilution System for Large Numbers of Broth Dilution Tests	96
Table 4.24. Comparison of Overnight to 3-Hour Reading with ICS Broth Dilution Method	100
Table 4.25. Comparison of Overnight MIC Readings with Inocula of 10^5 per ml to 3-Hour Readings with Inocula of 10^7 per ml	101
Table 4.26. Overall Discrepancies between Autobac I (Rapid Broth-Disc-Elution) and the NCCLS Disc Diffusion Test Results, for Gram-negative Bacilli and Enterococci	104
Table 4.27. Overnight Broth Tube Dilution Visual MICs with Inocula of 10^6 Organisms per ml Compared to 6-Hour Electrical Impedance MICs with Inocula of 10^7 Organisms per ml	108
Table 4.28. Dilution Factors Relating Radiometric to Broth Dilution Results	109
Table 5.1. Anaerobes Most Often Isolated from Clinically Significant Infections at the Mayo Clinic	115
Table 5.2. Incidence of Isolation of the <i>Bacteroides fragilis</i> "Group" from Clinical Specimens at the Mayo Clinic	115
Table 5.3. Incidence of Anaerobes Isolated from Significant Bacteremias at the Mayo Clinic	115
Table 5.4. Antibiotic Susceptibility of <i>Bacteroides fragilis</i> Isolated from Clinical Specimens at the Mayo Clinic	115
Table 5.5. Antibiotic Susceptibility of <i>Bacteroides</i> Other than <i>B. fragilis</i> "Group" Isolated at the Mayo Clinic	116
Table 5.6. Correlation of Penicillin MIC and β -Lactamase Activity (Slide Test) in Isolates of <i>Bacteroides melaninogenicus</i>	116
Table 5.7. Antibiotic Susceptibility of Fusobacterium Isolated from Clinical Specimens at the Mayo Clinic	117
Table 5.8. Antibiotic Susceptibility of Anaerobic Gram-positive Cocci Isolated from Clinical Specimens at the Mayo Clinic	117
Table 5.9. Antibiotic Susceptibility of Clostridium Isolated from Clinical Specimens at the Mayo Clinic	117
Table 5.10. Concentration of Antimicrobials Used in the Broth Disc Method of Susceptibility Testing	126
Table 5.11. Estimates of Antimicrobial Susceptibility of Anaerobes by Measurement of Inhibition Zone Diameters Obtained with Agar (Disc) Diffusion Method	127
Table 6.1. Drug Concentrations for Proportion Method of Susceptibility Testing Using Various Culture Media	138
Table 6.2. Direct Susceptibility Test-Proportion Method	139
Table 6.3. Dilution of Sputum Concentrate for Inoculation of Susceptibility Test Medium	139
Table 6.4. Indirect Susceptibility Test-Proportion Method	140
Table 6.5. Preparation of Inoculum for Indirect Proportion Method Susceptibility Test	140
Table 6.6. Reading and Reporting of Proportion Method Susceptibility Test Results	140
Table 6.7. Cumulative Percentages of Mycobacterial Strains Inhibited by Antituberculosis Drugs	144
Table 6.8. Absolute Concentration Method of Susceptibility Testing	145

Table 6.9.	Resistance Ratio Method of Susceptibility Testing	145
Table 6.10.	Drug Concentrations in Löwenstein-Jensen Medium for Absolute Concentration and Resistance Ratio Susceptibility Test Methods	145
Table 6.11.	Radiometric Method of Susceptibility Testing Utilizing ³ H-Uracil Incorporation	145
Table 6.12.	Criteria for Performance of Mycobacterial Susceptibility Tests	146
Table 6.13.	Proposed Mechanisms of Action of Antimycobacterial Drugs	147
Table 6.14.	Serum Levels of Antituberculosis Drugs	160
Table 6.15.	Procedures for Determining Serum Drug Levels	161
Table 6.16.	Proposed Levels of Laboratory Service	165
Table 7.1.	Antifungal Antibiotics	171
Table 7.2.	Test Procedures for Antifungal Antibiotics	171
Table 7.3.	Tube Dilution Susceptibility Test	175
Table 7.4.	Agar Well Diffusion Test	176
Table 7.5.	Disc Agar Diffusion Test	176
Table 7.6.	Choice of Antifungal Susceptibility Test	177
Table 7.7.	Minimal Inhibitory Concentration (MIC) of Amphotericin B against Some Pathogenic Fungi	177
Table 7.8.	Media Preparation/Ingredients	178
Table 7.9.	Radiometric Susceptibility Test Procedure	179
Table 7.10.	Drug Dilutions	180
Table 7.11.	Minimal Inhibitory Concentration (MIC) for Nystatin	180
Table 7.12.	Minimal Inhibitory Concentration (MIC) for Clotrimazole	181
Table 7.13.	Minimal Inhibitory Concentration (MIC) for Miconazole	182
Table 7.14.	Minimal Inhibitory Concentration (MIC) for 5-Fluorocytosine	182
Table 7.15.	Microtiter-Colorimetric Susceptibility Test	183
Table 7.16.	Assays for Amphotericin B	186
Table 9.1.	Standard Buffers for the Initial Solution and Subsequent Dilution of the Most Commonly Used Antibiotics	210
Table 9.2.	Standard Curve Concentrations for Commonly Utilized Antibiotics	213
Table 9.3.	Approximate Lower Limits of Detectability Utilizing <i>Staphylococcus aureus</i> ATCC 25923 and <i>Escherichia coli</i> ATCC 25922 as the Test Organisms	214
Table 9.4.	Conditions for the Assay of the Major Classes of Antibiotics by the Plate Diffusion Assay	215
Table 9.5.	Bibliography of Methods for the Assay of Antibacterial Agents and Chemotherapeutic Agents	216
Table 9.6.	Conditions for Performing Electrophoretic Analysis of Antibiotics	231
Table 9.7.	Flow Sheet	238
Table 9.8.	Cross-reactivity of Antibiotics with Antigentamicin Antibody	239
Table 9.9.	Specificity of the Adenylating Radioenzymatic Assay for Gentamicin	245
Table 9.10.	Comparison of the Microbiological, Radioenzymatic, and Radioimmunoassay Techniques for the Assay of Antibiotics	256
Table 9.11.	Average Attainable Blood Levels of Most Widely Used Antibiotics (Normal Adults)	258
Table 9.12.	Penetration of Antibiotics into Uninflamed, Unobstructed Compartments as a Percentage of the Blood Level	259
Table 9.13.	Effect of Dialysis on the Removal of Antibiotics from the Blood	259
Table 10.1.	Binding of Antimicrobials to Various Constituents of Tissue	267
Table 10.2.	Protein Binding of Antimicrobials in Human Serum or Plasma as Determined by Equilibrium Dialysis, Ultrafiltration, Ultracentrifugation and Microbiologic Methods	275
Table 10.3.	Protein Binding of Antimicrobials in Serum or Plasma of Various Animal Species	281
Table 11.1.	Dilution of an Antimicrobial for Checkerboard Testing in Broth	301
Table 11.2.	Dilution of an Antimicrobial for Checkerboard Testing in Agar	302
Table 11.3.	Calculation of the Fractional Inhibitory Concentration Index for Combinations of Two Antimicrobials	304
Table 11.4.	Comparison of Techniques Used to Assess Antimicrobial Combinations	308
Table 11.5.	Synergistic Antimicrobial Combinations <i>in Vitro</i>	332
Table 12.1.	Species, Treatments, and Number of CFU per Membrane Grown before and after Treatment and after Transfer to Drug-free Media	348
Table 12.2.	Number of Pneumococci Grown for 18 Hours at Various Concentrations of Methicillin	359

Table 12.3. Number of Pneumococci Recovered on Drug-free Agar	359
Table 12.4A. Effect of Antibiotics on Ribosome Frequency per Square Micrometer of Cross-Sectional Area of <i>Proteus mirabilis</i>	372
Table 12.4B. Comparison of Ribosome Counts (per Square Micrometer) by Two Methods	372
Table 12.5. MIC and MIC/MAC Ratio for <i>Escherichia coli</i>	395
Table 12.6. MIC and MIC/MAC Ratio for <i>Klebsiella pneumoniae</i>	396
Table 12.7. MIC and MIC/MAC Ratio for <i>Proteus mirabilis</i>	396
Table 12.8. MIC and MIC/MAC Ratio for <i>Pseudomonas aeruginosa</i>	397
Table 12.9. MIC and MIC/MAC Ratio for <i>Staphylococcus aureus</i>	398
Table 12.10. MIC and MIC/MAC Ratio for <i>Streptococcus faecalis</i>	399
Table 12.11. MIC and MIC/MAC Ratios for <i>Escherichia coli</i> and <i>Proteus mirabilis</i> , Simultaneous Test for Cephalothin and Cefamandole	399
Table 12.12. Synoptic Table with Average MIC/MAC Ratios for All Species and Drugs Tested	400
Table 12.13. MIC/MAC Ratios Obtained with Turbidimetric Method	400
Table 12.14. MIC/MAC Ratio of Ampicillin for Strains of <i>Escherichia coli</i> Sensitive (1-5) and Highly Resistant (R) to Ampicillin	400
Table 13.1. Expected Minimum Inhibitory Concentration (MIC) Endpoints for Quality Control of Dilution Antimicrobial Susceptibility Tests	415
Table 13.2. Theoretical Maximum Allowable Standard Deviation for a Probability Level of 0.01 of a Very Major or Major Interpretative Error	416
Table 15.1. Biochemical Mechanisms of Plasmid-mediated Resistance	434
Table 15.2. Change in Antibiotic Resistance of Salmonella Isolates over Period 1967-1975	438
Table 15.3. Some Molecular Properties of R Plasmids Found in Enteric Bacteria	440
Table 15.4. Comparison of Molecular Weight Determinations by Agarose Gel Electrophoresis and Contour Length Measurements of Plasmids from Enterotoxigenic <i>Escherichia coli</i>	447
Table 17.1. Classes of Aminoglycoside-Aminocyclitol Antibiotics	475
Table 17.2. Ribosomal Proteins Associated with the Interaction of Aminoglycoside-Aminocyclitol Antibiotics and Ribosomes	477
Table 17.3. Aminoglycoside-Aminocyclitol Substrates	780
Table 17.4. Typical Antibiotic Resistance Phenotypes Associated with Resistance Enzymes	482
Table 18.1. Markers Used in Epidemiologic Studies	492
Table 18.2. Antibacterial Agents Used for Taxonomy	494
Table 18.3. Group D Streptococci	495
Table 18.4. Selective Media Used for Screening Purposes	499
Table 19.1. Microorganisms Used for Primary Screening of New Antimicrobial Agents: One Strain of Each	508
Table 19.2. Microorganisms Used for Detection of Activity of Derivatives of Known Antibiotics: Primary Screen (Antibacterials Related to β -Lactam or Aminoglycoside Antibiotics)	509
Table 19.3. <i>In Vitro</i> Activity of Ampicillin Against Strains of Gram-negative Bacteria: Comparison of Disc Diffusion and Broth and Agar Dilution Test-Results	513
Table 19.4. Evaluation of Synergistic Potential of Mecillinam (an Amidinopenicillin) when Combined with Ampicillin (an Aminopenicillin) by Disc Diffusion and Agar and Broth Dilution Tests	516
Table 19.5. Comparison of Experimental PD ₅₀ Values for Penicillin G and Ampicillin with Clinical Dose	519
Table 19.6. Comparison of Experimental PD ₅₀ Values for Cephalothin and Tetracycline with Clinical Dose	519
Table 19.7. Comparison of Experimental PD ₅₀ Values for Amphotericin B with Clinical Dose	520
Table 19.8. Determination of Minimal Lethal Dose (MLD)	522
Table 19.9. Examples of Determination of 50% Protective Dose (PD ₅₀) Value from Results of Experiment in Which Mice Infected with 1000 MLDs of Pathogen Were Treated with Active Substance	523
Table 19.10. Effect of Treatment Regimen on Activity of Amoxicillin and Ampicillin when Administered Once Subcutaneously or Orally to Mice Infected with <i>Streptococcus pneumoniae</i> , Types 1 and 2	524
Table 19.11. Comparison of <i>In Vitro</i> Activities of Ampicillin and Gentamicin Against Gram-negative Bacterial Infections in Mice	524
Table 19.12. <i>In Vivo</i> and <i>In Vitro</i> Activity of Ampicillin Against Strains of <i>Serratia marcescens</i>	525
Table 19.13. Activity of Various Agents Against <i>Fusobacterium necrophorum</i> Infections in Mice	525
Table 19.14. Effect of Administering Ampicillin and Mecillinam Alone or Combined in Various Ratios Subcutaneously Against Gram-negative Bacterial Infections in Mice	529

Table 19.15. <i>In vivo</i> Activity of Myxin Against Systemic and Local Infections in Mice	530
Table 19.16. Effect of Route of Infection on Activity of Amoxicillin and Ampicillin Against <i>Streptococcus pneumoniae</i> Type 1	531
Table 19.17. Results of Therapeutic Trials of Cephalothin and Lincomycin for Treatment of Osteomyelitis in Rabbits	534
Table 19.18. Recommendations to Meet Federal Food, Drugs and Cosmetic Act Regulations for Drugs to be Given Under Medical Supervision	540
Table 19.19. Toxicologic Tests in Animals	540
Table 19.20. Schema for Grading Light and Electron Microscopic Proximal Tubule Changes	542
Table 19.21. Mutant Frequency of Five Compounds Tested in <i>Salmonella typhimurium</i> Host-mediated Assay System	543
Table 20.1. Labeled Contents of Antibiotic and Representative Chemotherapeutic Discs Manufactured for Use in the United States	550
Table 20.2. Concentrations of Drugs in Discs Used in Culture Media for Sensitivity Testing of Mycobacteria	551
Table 20.3. Solvents Usually Used in Manufacture of Discs and Prescribed by FDA for Preparation of Standard Response Lines for Assay	552
Table 20.4. Properties of Various Grades of Analytic Paper	554
Table 20.5. Antibiotic Standards Used by FDA for Assay and Control of Antibiotic Discs	556
Table 20.6. Antibiotics, Type of Market Disc, Stock Concentration, and Standard Disc Contents for Various Assays	559
Table 20.7. Error Variances of USP 3 × 3 Design Assays, Using Different Pipetting Systems	560
Table 20.8. Relative Potencies Obtained from Different Aliquots Dispensed to Discs	560
Table 20.9. Stability of Control Discs Prepared with Various U.S. Antibiotic Reference Standards	561
Table 20.10. Expected Zone Diameters and Dose Response Slopes Promoted by Suitable Disc Assay Media	563
Table 20.11. Strains of Microorganisms Employed in Assay of Antibiotic Discs	564
Table 20.12. Expected Ranges of Zone Diameters and Precision of Various Antibiotic Disc Assays	568
Table 20.13. Activity of Diagnostic Sensitivity Powders	570
Table 21.1. Determination of Antiviral Activity in Cell Culture	574
Table 21.2. Cell Culture Assays for Interferon	579
Table 21.3. Composition of Eagle's Minimum Essential Medium	582
Table 21.4. Phosphate-buffered Saline (PBS) (pH 7.4)	582
Table 21.5. Trypsin-EDTA Solution	582
Table 22.1. Urinary Excretion of Certain Antimicrobial Agents	593
Table 23.1. Incidence of Microbial Flora Isolated from Hospital Patients in the United States: Species Isolated from All Body Sites, January 1971—May 1978	611
Table 23.2. Trend of Incidence of Microbial Flora Isolated from Hospital Patients in the United States, 1973–1978	611
Table 23.3. Trend of Incidence of Microbial Flora from Urine, 1973–1977	613
Table 23.4. Trend of Incidence of Microbial Flora from Blood, 1973–1977	614
Table 23.5. Trend of Incidence of Microbial Flora from Cerebrospinal Fluid, 1973–1977	615
Table 23.6. Trend of Incidence of Microbial Flora from Wounds, 1973–1977	616
Table 23.7. Trend of Antibiotic Susceptibility of Microbial Flora Isolated from Hospital Patients in the United States, 1973–1977: Gram-negative	617
Table 23.8. Trend of Antibiotic Susceptibility of Microbial Flora Isolated from Hospital Patients in the United States, 1973–1977: Gram-positive	619
Table 23.9. 1977 Antibiotic Susceptibility: Anaerobes	620
Table 23.10A–D. Antibiotic Susceptibility of Microbial Flora: Organisms and Antibiotics in Alphabetical Order	621
Table 23.11. Minimum Inhibitory Concentrations (MIC): Range and Partial Cumulative Percentage Inhibited	633
Table 23.12. Cumulative Percentage MIC of Frequently Isolated Bacteria Inhibited by Aminoglycosides, Penicillins, Cephalosporins, and Other Antimicrobial Agents	686
Table 23.13. Cumulative Percentage of Anaerobic Bacteria Inhibited by Antibiotics	705
Table 23.14. Activity of β -Lactamase-labile Antibiotics in Presence of Sodium Clavulanate	711

Procedure for Testing Antibiotics in Agar Media: Theoretical Considerations

ARTHUR L. BARRY, Ph.D.

Introduction	1	Dynamics of Zone Formation	11
Nature of Agar	2	Critical Concentration	11
Alternative Gelling Agents	2	Critical Time	14
Interaction between Antimicrobial Agents and Agar	3	Critical Population	15
Microbial Growth on an Agar Medium	5	Characteristics of the Zone Edge	16
Agar Dilution Susceptibility Tests	6	Factors Influencing Diffusion Tests	17
Selection of Agar Medium	6	Inoculum Density	17
Preparation of Antimicrobial Plates	6	Nature of the Zone Edge	17
Standardization of Inoculum	7	Agar Depth	17
Inoculation of Test Plates	7	Composition of the Agar Medium	18
Incubation of Test Plates	7	Growth Characteristics of the Test Strain	18
Agar vs. Broth Dilution Methods	8	Temperature of Incubation	18
Agar Diffusion Procedures	9	Incubation Time	18
Antimicrobial Diffusion	9	Timing of Drug Application	18
Application of Drug Solutions to Agar Medium	9	Concentration of Antimicrobial in the Reservoir	19
Diffusion Through an Agar Gel	10	Presence of Serum Proteins	19
		Presence of Two or More Drugs in the Reservoir	21

INTRODUCTION

Laboratory procedures involving antimicrobial agents are commonly performed in agar media. To determine microbial susceptibility to an antimicrobial agent, measured amounts of drug may be incorporated into an agar medium which is then inoculated with the microorganism to be tested. After appropriate incubation, one can determine whether the microorganism is capable of growing in the presence of that concentration of antimicrobial. When a series of petri plates are prepared with varying amounts of antimicrobial, one can determine the minimal concentration required to inhibit growth of the microorganism (the minimal inhibitory concentration or MIC). Such drug dilution tests carried out in agar media are commonly referred to as agar dilution procedures, as opposed to broth dilution procedures which are carried out in a broth medium.

Rather than preparing dilutions of each drug, antimicrobial susceptibility tests may be performed by agar diffusion methods. A single concentration of

antimicrobial is applied to a reservoir on a seeded agar medium and the drug is allowed to diffuse into the surrounding medium. This exposes the test organism to a continuous gradient of drug concentrations, with diminishing concentrations at increasing distances from the reservoir. Agar diffusion techniques have also been adapted for measuring the concentration of antimicrobial in body fluids or other material (bioassay procedures). Although performed for entirely different purposes, bioassay and susceptibility tests are both agar diffusion procedures which are governed by the same theoretical principles.

The purpose of this chapter is to review theoretical considerations governing *in vitro* tests of antimicrobial agents in agar media. Both dilution and diffusion techniques will be reviewed in principle. Specific details of agar diffusion susceptibility and bioassay procedures are covered in Chapters 2 and 9. Antimicrobial tests performed in broth media are discussed in Chapter 4.

THE NATURE OF AGAR

Almost any nutrient broth can be solidified by adding 1.5–2.0% agar—a complex polysaccharide substance prepared from one of several species of red seaweed, the *Rhodophyceae*. The physical characteristics of agar which make it useful for microbiological work is its unique gelling capacity. It is liquid when heated to boiling temperatures and remains liquid when cooled to 45–50°C. At 30–45°C, it forms a firm gel and once gelled it does not liquefy until heated to near boiling temperatures again. Agar itself is essentially unaffected by microbial growth and thus it is ideally suited for solidifying many nutrient media.

Agar is a complex substance which is yet to be completely characterized and chemically synthesized. Because agar is a natural product, one would expect some lot-to-lot variability in the actual composition of different agars. The quality of a batch of agar will depend upon the method of extraction from the seaweed, the quality of the seaweed itself and the environment in which the seaweed is grown. Agar contains at least two types of polysaccharides; agarose and agarpectin. Agarose is essentially neutral, with few ionic charges. Most agars carry an overall negative charge, largely due to acid and sulfate groups on the polysaccharides. Normally a variety of metallic cations are bound to the negatively charged radicals on the polysaccharide. The concentration of such cations can be reduced by dialysis or by washing the agar with large volumes of water (22). Traces of calcium are necessary in order to maintain the ability of the agar to gel. In addition to cations, agars often contain other trace elements which may be either stimulatory or inhibitory to microbial growth. Complete removal of all contaminating substances might actually have a deleterious effect on the growth of some microorganisms and might stimulate the growth of other microorganisms. In the absence of a completely defined synthetic gelling agent, microbiological work in agar media is not likely to be extremely well standardized or completely reproducible.

Alternative Gelling Agents

Because of the many problems inherent in manufacturing consistently uniform agar media, alternative gelling agents deserve consideration. A chemically defined, synthetic product would be ideal if it is not affected by microbial growth and does not affect the activity of antimicrobial agents.

Separan NP 10 is one such substance which may reduce the amount of agar needed to produce a satisfactory gel. It is a high molecular weight polymer produced by polymerization of acrylamide. It is essentially nonionic in solution but has a preponderance of amide groups, a small proportion of which are hydrolyzed to anionic carboxyl groupings. The vis-

cous polymer can be added to 0.5% agar to obtain a gel comparable to that obtained with 1% agar alone (30).

Another potentially useful substitute for agar is a polyoxyethylene polymer which was developed by Union Carbide (Tuxedo, New York) and has been given the trade name Neutra-Gel. It is a synthetic polymer which is converted to the polymer in standard plastic petri plates by a proprietary cross-linking process which produces carbon-carbon covalent bonds. The chemical structure is $(CH_2-CH_2-O)_n$. It is an inert, optically clear, stable, irreversible solid which is not affected by extremes in temperature.

Neutra-Gel is supplied as a presterilized gel which is tightly bonded to a plastic petri plate. It contains only distilled water, traces of ionic materials and the polymer. The amount of water and ionized materials can be adjusted at the time of manufacture, depending upon the needs of the user. For use, a 2–3 times concentrated nutrient broth is aseptically added to the hydrogel and then allowed to absorb into the hydrogel by standing several hours, preferably overnight. The appropriate concentration of nutrients in the broth will be provided when the concentrated broth is diluted by the water in the hydrogel layer. Only solubilized materials will be incorporated into the polymer, particulate substances will remain on the surface. The starch in some batches of Mueller-Hinton broth is not completely soluble and will tend to form a cloudy film over the surface of the hydrogel. Consequently, susceptibility testing on a Mueller-Hinton medium solidified with hydrogel is not entirely satisfactory.

By incorporating the synthetic amino acid medium (SAAM) (23) into the synthetic hydrogel, a completely defined medium can be prepared. The possibility of using such a defined medium for antimicrobial susceptibility testing has been investigated (16a, 27).

Table 1.1 includes the results of diffusion tests on Mueller-Hinton agars from three different manufacturers and on SAAM solidified with 0.9% Ionagar and with the synthetic hydrogel. The effect on zone sizes varied with the organism and antimicrobial agent being tested. Variability in the performance of Mueller-Hinton agars from different manufacturers has been shown to be statistically significant (6). On SAAM agar, the zones were generally larger than on Mueller-Hinton agar and the differences were especially marked when *Staphylococcus aureus* was being tested. The polymyxins were the major exceptions, they both gave zones which were essentially the same size on either agar medium. On the neutral polymer, the polymyxins gave much larger zones than on the agar media. Presumably, diffusion of the cationic polymyxin molecules is inhibited by agar but not by

Table 1.1

Mean Zone Sizes on Mueller-Hinton Agars from Three Commercial Sources and on a Synthetic Amino Acid Medium (SAAM) Solidified with 0.9% Ionagar and with a Synthetic Hydrogel (Neutra-Gel), All Inoculated by a Flooding Technique on Six Separate Drugs

Test Strain	Medium	Mean Zone Diameters (mm) Seven Replicates Each Drug									
		Polymyxin B	Colistin	Gentamicin	Streptomycin	Nitrofurantoin	Tetracycline	Cephalothin	Ampicillin	Carbenicillin	Chloramphenicol
<i>Staphylococcus aureus</i>	Mueller-Hinton agar:										
	Difco	9.4	6 ^b	23.6	17.9	20.6	25.6	31.7	30.4	32.4	23.9
	Pfizer	10.0	6	23.7	19.7	20.5	26.6	32.1	30.4	33.1	23.6
	BBL	9.7	6	24.1	18.9	21.3	25.3	31.7	30.0	33.0	23.3
	SAAM										
	Ionagar	9.3	6	29.7	20.0	30.8	31.8	38.5	46.3	47.4	28.5
	Hydrogel	12.1	6	30.9	19.0	22.8	27.7	41.7	36.9	43.6	22.4
<i>Escherichia coli</i>	Mueller-Hinton agar										
	Difco	16.6	16.0	21.6	16.1	22.4	21.0	19.3	16.3	24.4	23.4
	Pfizer	13.9	13.3	21.4	16.7	23.1	22.9	23.1	18.1	25.9	24.5
	BBL	13.7	13.0	19.1	14.9	22.1	21.7	19.9	17.3	24.0	22.9
	SAAM										
	Ionagar	13.8	13.4	25.0	19.3	24.6	22.4	20.7	14.7	24.6	24.4
	Hydrogel	18.5	16.6	25.3	17.0	19.8	18.6	18.0	14.0	21.1	21.0
<i>Pseudomonas aeruginosa</i>	Mueller-Hinton agar:										
	Difco	16.9	14.9	23.3	14.9	6	9.4	6	6	29.1	14.9
	Pfizer	13.3	11.7	21.6	15.9	6	8.7	6	6	29.1	15.3
	BBL	14.1	13.0	23.6	16.7	6	10.3	6	6	31.7	16.0
	SAAM										
	Ionagar	15.1	13.9	23.7	9.7	6	11.7	6	6	30.0	15.4
	Hydrogel	20.6	17.6	25.4	10.3	6	8.7	6	6	25.1	14.0

^a Adapted from Barry and Effinger (6, 6a)

^b 6 = no zone of inhibition around the 6-mm disc

the inert hydrogel. Diffusion of the other antimicrobics through the hydrogel appears to be a little slower since the zones tend to be slightly smaller.

Since the SAAM-hydrogel medium is a completely defined synthetic medium, there should be a minimal amount of variation in the performance of different batches. Precision of diffusion tests in the SAAM-hydrogel medium is compared to that on a single lot of Mueller-Hinton agar (Difco, Control No. 579660) by the control data summarized in Table 1.2. Fifteen different determinations were made for each antimicrobial during a 4-month study period. The precision of tests on the SAAM-hydrogel medium was definitely inferior to the precision of tests on Mueller-Hinton agar, especially when the *S. aureus* control strain was being tested. In part, the variability in results of tests on SAAM-hydrogel plates reflects the relatively poor growth of *S. aureus* on SAAM. *Escherichia coli* provided satisfactory growth on both media and the precision of diffusion tests on the two media was more nearly comparable.

In summary, the synthetic polymer can be used to solidify a synthetic amino acid medium and the solid

medium can be used for diffusion tests. The results of diffusion tests on such a chemically defined medium will differ from that on Mueller-Hinton agar and the differences will vary with the antimicrobial and type of microorganism being tested. At this point, it is only possible to document differences in results; it is not possible to decide which result is the "correct" one. Since the synthetic hydrogel is not yet available commercially, it appears that the use of agar media will continue for some time. Consequently, we should develop an appreciation for the ways in which agar affects antimicrobial agents.

Interaction between Antimicrobial Agents and Agar

Antimicrobics with cationic molecular structures may be electrostatically bound to acid or sulfate groups on bacteriologic agar and consequently the rate of diffusion through the agar gel is diminished. The polymyxins are examples of fairly large, strongly cationic molecules which diffuse at a slow rate and produce relatively small zones of inhibition with agar

Table 1.2

Precision of Diffusion Tests on Mueller-Hinton Agar (MHA) and on a Synthetic Amino Acid Medium (SAAM) Solidified with a Synthetic Polymer (Neutra-Gel), Expressed as Standard Deviations Calculated from 16 Replicate Tests^a

Antimicrobial	Standard Deviation in Zone Diameters (mm)					
	<i>Staphylococcus aureus</i>		<i>Escherichia coli</i>		<i>Pseudomonas aeruginosa</i>	
	SAAM	MHA	SAAM	MHA	SAAM	MHA
Gentamicin	2.82 ^b	0.89 ^b	1.17	0.97	1.54	2.02
Streptomycin	0.84 ^b	0.61 ^b	1.06	0.78	1.65	1.98
Polymyxin B	2.03	0.77	0.86	0.41	1.60	0.49
Colistin	NZ ^c	NZ	0.62	0.36	1.68	0.52
Chloramphenicol	1.88	1.51	0.93	1.06	1.92	2.33
Tetracycline	2.40	1.53	1.17	1.19	2.02	1.35
Doxycycline	1.91	1.35	1.59	1.81	1.12	1.35
Nitrofurantoin	2.17	0.93	1.05	0.62	NZ	NZ
Carbenicillin	1.41 ^b	1.98 ^b	0.66	0.96	2.43	3.13
Ampicillin	2.41 ^b	1.72 ^b	1.25	1.62	NZ	NZ
Cephalothin	4.62	1.59	1.13	1.12	NZ	NZ

^a Adapted from A. L. Barry and L. J. Effinger (6a).

^b Standard deviations based on 8 replicates, rather than 16 tests.

^c NZ = no zone of inhibition.

diffusion tests. Much larger zones on inhibition can be obtained with the polymyxins, if the agar is treated with protamine to block the acidic sulfate groups (26). Other drugs, such as the aminoglycosides might have a similar tendency for electrostatic binding to active groups on the agar molecule. The polymyxins and aminoglycosides tend to give larger zones of inhibition when tested in agarose (26, 33). The polymyxins produced much larger zones on a neutral polymer than on agar media (Table 1.1).

Calcium, magnesium, zinc and other cations are often found associated with bacteriologic agars. There is a great deal of variability in the cation content of different lots of Mueller-Hinton agars (Table 1.3). The concentration of calcium, magnesium and other divalent cations profoundly affects the activity of the aminoglycosides against *Pseudomonas* spp. For example, one strain of *Pseudomonas aeruginosa*, tested on the 14 lots of Mueller-Hinton agar described in Table 1.3, gave gentamicin MICs ranging from 0.5 to 6.0 µg/ml, tobramycin MICs ranging from 0.2 to 2.0 µg/ml and amikacin MICs varied from 0.8 to 11 µg/ml (42). The activity of the aminoglycosides against bacteria other than *Pseudomonas* sp. is not affected as dramatically by the calcium and magnesium ions in the agar medium. The concentration of cations in the culture medium also affects the activity of polymyxin B and tetracycline against *Pseudomonas* spp. (17). Mueller-Hinton agar is rich in phosphates. Magnesium and calcium ions tend to form stable polyphosphate complexes, especially when the agar medium is autoclaved. Antimicrobials are affected more by the cations which are free to ionize than by those that are bound in polyphosphates or other complexes. For that reason the data in Table 1.3 might be a little

Table 1.3
Divalent Cation Content of 14 Different Lots of Mueller-Hinton Agar from Three Manufacturers (4–5 Lots from Each)^a

Cation	Cation Concentration (µg/ml) ^b		
	Minimum	Maximum	Mean
Calcium	6.58	85.01	50.51
Magnesium	2.89	46.42	19.83
Copper	0.068	0.665	0.141
Zinc	0.285	1.220	0.662
Iron	0.551	4.030	1.587

^a Data adapted from Washington et al. (42).

^b Constituted medium (µg/ml) as determined by atomic absorption spectrophotometry after wet-ashing with nitric and perchloric acid.

misleading since the measurements reflect both free and bound cation.

The mechanism by which metallic cations influence the activity of antimicrobial agents is not entirely understood. The most likely explanation lies in the observation that divalent cations may be essential for maintaining structural integrity of the cell wall. The lipopolysaccharides in the cell wall of *P. aeruginosa* are probably stabilized by cross-linking with divalent cations. Removal of the cell wall cations may increase the permeability to a variety of unrelated molecules. When cells of *P. aeruginosa* are grown in media with abundant amounts of calcium and magnesium, the cations should be incorporated into the cell wall thus making it less permeable to aminoglycoside drugs and to other compounds. Consequently, the microorganism would require somewhat greater concentrations of drug for inhibition. Alternatively, the cations may compete with the drug for binding sites on or in the