

METHODS IN MICROBIOLOGY

VOLUME 5A

METHODS in MICROBIOLOGY

Edited by

J. R. NORRIS

*Borden Microbiological Laboratory,
Shell Research Limited,
Sittingbourne, Kent, England*

D. W. RIBBONS

*Department of Biochemistry,
University of Miami School of Medicine,
and Howard Hughes Medical Institute,
Miami, Florida, U.S.A.*

Volume 5A



1971

ACADEMIC PRESS
London and New York

ACADEMIC PRESS INC. (LONDON) LTD
Berkeley Square House
Berkeley Square
London, W1X 6BA

U.S. Edition published by
ACADEMIC PRESS INC.
111 Fifth Avenue
New York, New York 10003

Copyright © 1971 by ACADEMIC PRESS INC. (LONDON) LTD

All Rights Reserved

No part of this book may be reproduced in any form by photostat, microfilm, or any other means, without written permission from the publishers

Library of Congress Catalog Card Number: 68-57745
ISBN: 0-12-521505-3

PRINTED IN GREAT BRITAIN BY
ADLARD AND SON LIMITED
BARTHOLOMEW PRESS, DORKING

LIST OF CONTRIBUTORS

- IRENE BATTY, *Wellcome Research Laboratories, Beckenham, Kent, England*
J. DE LEY, *Laboratory for Microbiology, Faculty of Sciences, State University, Gent, Belgium*
M. ENOMOTO, *National Institute of Genetics, Mishima, Shizuoka-ken, Japan*
T. IINO, *National Institute of Genetics, Mishima, Shizuoka-ken, Japan*
SUSAN M. JONES, *Division of Agricultural Bacteriology, University of Aberdeen, Scotland*
W. H. KINGHAM, *Shell Research Limited, Milstead Laboratory of Chemical Enzymology, Sittingbourne, Kent, England*
H. KOFFLER, *Department of Biological Sciences, Purdue University, Lafayette, Indiana, U.S.A.*
J. E. M. MIDGLEY, *Department of Biochemistry, University of Newcastle-upon-Tyne, England*
J. R. NORRIS, *Shell Research Limited, Borden Microbiological Laboratory, Sittingbourne, Kent, England*
C. L. OAKLEY, *Department of Bacteriology, The Medical School, Leeds, England*
A. M. PATON, *Division of Agricultural Bacteriology, University of Aberdeen, Scotland*
L. B. QUESNEL, *Department of Bacteriology and Virology, University of Manchester, Manchester, England*
R. W. SMITH, *Department of Biological Sciences, Purdue University, Lafayette, Indiana, U.S.A.*
HELEN SWAIN, *Milstead Laboratory of Chemical Enzymology, Sittingbourne, Kent, England*
R. O. THOMPSON, *The Wellcome Research Laboratories, Langley Court, Beckenham, Kent, England*
P. D. WALKER, *The Wellcome Research Laboratories, Langley Court, Beckenham, Kent, England*
ELIZABETH WORK, *Department of Biochemistry, Imperial College of Science and Technology, London, S.W.7, England*

ACKNOWLEDGMENTS

For permission to reproduce, in whole or in part, certain figures and diagrams we are grateful to the following—

Edward Arnold Ltd., London; Butterworths, London; Chapman & Hall, London; E. Leitz, Wetzlar; Longmans, Green & Co., London; Nippon Kogaku, Tokyo; Vickers Instruments, York, England; Wild Heebrugg, Switzerland; Karl Zeiss, Oberkochen, W. Germany.

Detailed acknowledgments are given in the legends to figures.

PREFACE

Volume 5 of "Methods in Microbiology" is concerned with the microbial cell—methods of observing it, of studying its properties and behaviour, of analysing it chemically and immunologically, and of purifying and characterizing its various "organelles" and macro-molecular components. Wherever possible, the emphasis has been placed on quantitative methods.

We have tried to cover relatively new techniques such as reflectance spectrophotometry, isoelectric focusing and polyacrylamide gel electrophoresis which appear to us to have considerable future potential in microbiology in addition to more generally used techniques such as those for cell disintegration and hybridization of nucleic acids which are not fully described in a concise form elsewhere.

As with earlier Volumes in the Series we have left the treatment of the different topics largely to the individual authors, restricting our editorial activity to ensuring consistency and avoiding overlaps and gaps between the contributions.

As contributions accumulated it became obvious that there was too much material for a single Volume and the content was divided. Volume 5A contains Chapters concerned with the direct observation or study of whole cells or organelles while Volume 5B is concerned with the disintegration of cells, their chemical analysis and the techniques used to separate and characterize their components.

Our thanks are due to the pleasant way in which our authors have co-operated with us and particularly to those who agreed to update their contributions when delay in the publication process made it necessary.

J. R. NORRIS
D. W. RIBBONS

April, 1971

CONTENTS OF PUBLISHED VOLUMES

Volume 1

- E. C. ELLIOTT AND D. L. GEORGALA. Sources, Handling and Storage of Media and Equipment
- R. BROOKES. Properties of Materials Suitable for the Cultivation and Handling of Micro-organisms
- G. SYKES. Methods and Equipment for Sterilization of Laboratory Apparatus and Media
- R. ELSWORTH. Treatment of Process Air for Deep Culture
- J. J. McDADE, G. B. PHILLIPS, H. D. SIVINSKI AND W. J. WHITFIELD. Principles and Applications of Laminar-flow Devices
- H. M. DARLOW. Safety in the Microbiological Laboratory
- J. G. MULVANY. Membrane Filter Techniques in Microbiology
- C. T. CALAM. The Culture of Micro-organisms in Liquid Medium
- CHARLES E. HELMSTETTER. Methods for Studying the Microbial Division Cycle
- LOUIS B. QUESNEL. Methods of Microculture
- R. C. CODNER. Solid and Solidified Growth Media in Microbiology
- K. I. JOHNSTONE. The Isolation and Cultivation of Single Organisms
- N. BLAKEBROUGH. Design of Laboratory Fermenters
- K. SARGEANT. The Deep Culture of Bacteriophage
- M. F. MALLETTE. Evaluation of Growth by Physical and Chemical Means
- C. T. CALAM. The Evaluation of Mycelial Growth
- H. E. KUBITSCHK. Counting and Sizing Micro-organisms with the Coulter Counter
- J. R. POSTGATE. Viable Counts and Viability
- A. H. STOUTHAMER. Determination and Significance of Molar Growth Yields

Volume 2

- D. G. MACLENNAN. Principles of Automatic Measurement and Control of Fermentation Growth Parameters
- J. W. PATCHING AND A. H. ROSE. The Effects and Control of Temperature
- A. L. S. MUNRO. Measurement and Control of pH Values
- H.-E. JACOB. Redox Potential
- D. E. BROWN. Aeration in the Submerged Culture of Micro-organisms
- D. FREEDMAN. The Shaker in Bioengineering
- J. BRYANT. Anti-foam Agents
- N. G. CARR. Production and Measurement of Photosynthetically Useable Light
- R. ELSWORTH. The Measurement of Oxygen Absorption and Carbon Dioxide Evolution in Stirred Deep Cultures
- G. A. PLATON. Flow Measurement and Control
- RICHARD Y. MORITA. Application of Hydrostatic Pressure to Microbial Cultures
- D. W. TEMPEST. The Continuous Cultivation of Micro-organisms: 1. Theory of the Chemostat
- C. G. T. EVANS, D. HERBERT AND D. W. TEMPEST. The Continuous Cultivation of Micro-organisms: 2. Construction of a Chemostat

J. RIČICA. Multi-stage Systems

R. J. MUNSON. Turbidostats

R. O. THOMSON AND W. H. FOSTER. Harvesting and Clarification of Cultures—
Storage of Harvest

Volume 3A

S. P. LAPAGE, JEAN E. SHELTON AND T. G. MITCHELL. Media for the Maintenance and Preservation of Bacteria

S. P. LAPAGE, JEAN E. SHELTON, T. G. MITCHELL AND A. R. MACKENZIE. Culture Collections and the Preservation of Bacteria

E. Y. BRIDSON AND A. BRECKER. Design and Formulation of Microbial Culture Media

D. W. RIBBONS. Quantitative Relationships Between Growth Media Constituents and Cellular Yields and Composition

H. VELDKAMP. Enrichment Cultures of Prokaryotic Organisms

DAVID A. HOPWOOD. The Isolation of Mutants

C. T. CALAM. Improvement of Micro-organisms by Mutation, Hybridization and Selection

Volume 3B

VERA G. COLLINS. Isolation, Cultivation and Maintenance of Autotrophs

N. G. CARR. Growth of Phototrophic Bacteria and Blue-Green Algae

A. T. WILLIS. Techniques for the Study of Anaerobic, Spore-forming Bacteria

R. E. HUNGATE. A Roll Tube Method for Cultivation of Strict Anaerobes

P. N. HOBSON. Rumen Bacteria

ELLA M. BARNES. Methods for the Gram-negative Non-sporing Anaerobes

T. D. BROCK AND A. H. ROSE. Psychrophiles and Thermophiles

N. E. GIBBONS. Isolation, Growth and Requirements of Halophilic Bacteria

JOHN E. PETERSON. Isolation, Cultivation and Maintenance of the Myxobacteria

R. J. FALLON AND P. WHITTLESTONE. Isolation, Cultivation and Maintenance of Mycoplasmas

M. R. DROOP. Algae

EVE BILLING. Isolation, Growth and Preservation of Bacteriophages

Volume 4

C. BOOTH. Introduction to General Methods

C. BOOTH. Fungal Culture Media

D. M. DRING. Techniques for Microscopic Preparation

AGNES H. S. ONIONS. Preservation of Fungi

F. W. BEECH AND R. R. DAVENPORT. Isolation, Purification and Maintenance of Yeasts

MISS G. M. WATERHOUSE. Phycomycetes

E. PUNITHALINGHAM. Basidiomycetes: Heterobasidiomycetidae

ROY WATLING. Basidiomycetes: Homobasidiomycetidae

M. J. CARLILE. Myxomycetes and other Slime Moulds

D. H. S. RICHARDSON. Lichens

- S. T. WILLIAMS AND T. CROSS. Actinomycetes
 E. B. GARETH JONES. Aquatic Fungi
 R. R. DAVIES. Air Sampling for Fungi, Pollens and Bacteria
 GEORGE L. BARRON. Soil Fungi
 PHYLLIS M. STOCKDALE. Fungi Pathogenic for Man and Animals: 1. Diseases of the Keratinized Tissues
 HELEN R. BUCKLEY. Fungi Pathogenic for Man and Animals: 2. The Subcutaneous and Deep-seated Mycoses
 J. L. JINKS AND J. CROFT. Methods Used for Genetical Studies in Mycology
 R. L. LUCAS. Autoradiographic Techniques in Mycology
 T. F. PREECE. Fluorescent Techniques in Mycology
 G. N. GREENHALGH AND L. V. EVANS. Electron Microscopy
 ROY WATLING. Chemical Tests in Agaricology
 T. F. PREECE. Immunological Techniques in Mycology
 CHARLES M. LEACH. A Practical Guide to the Effects of Visible and Ultraviolet Light on Fungi
 JULIO R. VILLANUEVA AND ISABEL GARCIA ACHA. Production and Use of Fungal Protoplasts

CONTENTS

LIST OF CONTRIBUTORS	v
ACKNOWLEDGMENTS	vi
PREFACE	vii
CONTENTS OF PUBLISHED VOLUMES	xi
Chapter I. Microscopy and Micrometry—L. B. QUESNEL	1
Chapter II. Staining Bacteria—J. R. NORRIS AND HELEN SWAIN	105
Chapter III. Techniques Involving Optical Brightening Agents— A. M. PATON AND SUSAN M. JONES	135
Chapter IV. Motility—T. IINO AND M. ENOMOTO	145
Chapter V. Production and Isolation of Flagella—R. W. SMITH AND H. KOFFLER	165
Chapter VI. Antigen-antibody Reactions in Microbiology— C. L. OAKLEY	173
Chapter VII. The Localization of Bacterial Antigens by the use of the Fluorescent and Ferritin Labelled Antibody Techniques— P. D. WALKER, IRENE BATTY AND R. O. THOMSON	219
Chapter VIII. Toxin-antitoxin Assay—IRENE BATTY	255
Chapter IX. Techniques for Handling Animals—W. H. KINGHAM	281
Chapter X. The Determination of the Molecular Weight of DNA Per Bacterial Nucleoid.—J. DE LEY	301
Chapter XI. Hybridization of DNA—J. DE LEY	311
Chapter XII. Hybridization of Microbial RNA and DNA— J. E. M. MIDGLEY	331
Chapter XIII. Cell Walls—ELIZABETH WORK	361
AUTHOR INDEX	419
SUBJECT INDEX	429

CHAPTER I

Microscopy and Micrometry

LOUIS B. QUESNEL

*Department of Bacteriology and Virology, University of Manchester,
Manchester, England*

I. Introduction	2
II. Light and its Behaviour	2
A. Theories of light	5
B. Light waves	7
C. Plane waves	8
D. Interference	10
E. Phase	10
F. Diffraction	11
G. Refraction and reflection	16
H. Filtration	18
I. Amplitude specimens and phase specimens	18
III. The Light Microscope—Bright-field Use	21
A. Image formation	23
B. Abbe theory	24
C. Magnification	26
D. Resolution and numerical aperture	27
E. Useful magnification of the microscope	32
F. Depth of focus	33
G. Illumination for microscopy	35
H. Imaging errors	40
I. Objectives	45
J. Eyepieces	48
K. Condensers	53
IV. Setting Up and Efficient Use of the Microscope	55
A. Critical illumination	56
B. Kohler illumination	57
V. Phase Contrast Microscopy	60
A. Basic conditions for phase contrast imaging	60
B. The phase plate and image quality	64
C. Condensers for phase contrast	68
VI. Setting-up and Use of a Phase Contrast System	70
A. Practical procedure	70
B. Immersion media and phase contrast	73
VII. Dark-field Illumination	75
VIII. Setting-up for Dark-field Illumination	78

IX. Filters and Their Use	81
A. Types of filter	81
X. Micrometry	88
A. Measurements on objects	88
XI. Microscopical Journals	101
XII. British Standards	101
XIII. Some Microscope Manufacturers	101
References	102

I. INTRODUCTION

The microscope is an essential tool for the microbiologist; without it he is working in the dark. Modern research microscopes are precision instruments designed with great skill and dedication, yet they are used by many in an indifferent, almost haphazard, way. It is impossible in the space of a single Chapter either to study the microscope historically or to study the scientific theory which lies behind its design, but only to provide the basic knowledge required for the intelligent and accomplished use of the instrument as a tool of microbiological investigation. Basic theory will be discussed only in so far as it is required for the realization of the full design potential of the modern microscope.

The main features of a modern microscope are illustrated in Fig. 1.

II. LIGHT AND ITS BEHAVIOUR

The visible spectrum used in light microscopy forms a very small portion of a much larger spectrum of electromagnetic radiations. This restricted spectrum is of particular use in microscopy because the biological sensitivity of the eye to radiations in this range enables us to use the brain as the "recording" or "display" stage of the process, albeit a temporary record. This does not mean that other sections of the electromagnetic spectrum cannot be used for microscopy—they can—but when invisible radiations are used in the image-forming process a display which "generates" wavelengths in the visible range must be used, e.g. photographic emulsions in U.V. microscopy, fluorescent screens in electron microscopy. The relationship between the visible spectrum and the electromagnetic spectrum (E.M.) is shown in Fig. 2. While the E.M. spectrum includes wavelengths of radiations from about 3000 metres to less than 10^{-12} centimetres visible light fills the very small range from about 7×10^{-5} cm down to 4×10^{-5} cm, or, in the units usually used to define wavelengths, from 7000 Angstrom units (\AA) to 4000 which is equivalent to 700 nanometers (nm) to 400 nm ($1 \text{\AA} = 10^{-8}$ cm; $1 \text{ nm} = 10^{-7}$ cm).

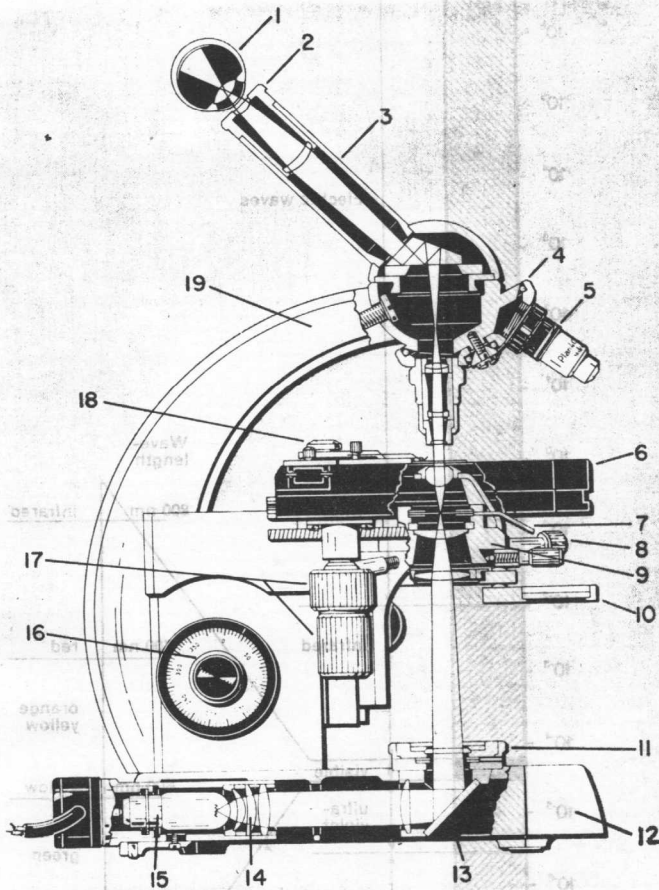


FIG. 1. A modern microscope with inclined oculars and built-in lamp—the Zeiss (Oberkochen) Standard RA Routine and Research Microscope. (Figure by courtesy of Carl Zeiss, Oberkochen, W. Germany.)

- | | |
|--|--|
| 1. Eye | 11. Diaphragm insert |
| 2. Eyepiece | 12. Base |
| 3. Eyepiece tube | 13. Mirror |
| 4. Revolving nosepiece | 14. Lamp collector lens system |
| 5. Objective | 15. Built-in low voltage lamp |
| 6. Mechanical stage | 16. Concentric coarse- and fine-focus control knobs |
| 7. Condenser diaphragm—control lever | 17. Concentric x-, y-axis control knobs for slide holder movements |
| 8. Control knob for swing-out top element of condenser | 18. Slide holder |
| 9. Condenser | 19. Limb |
| 10. Filter carrier | |

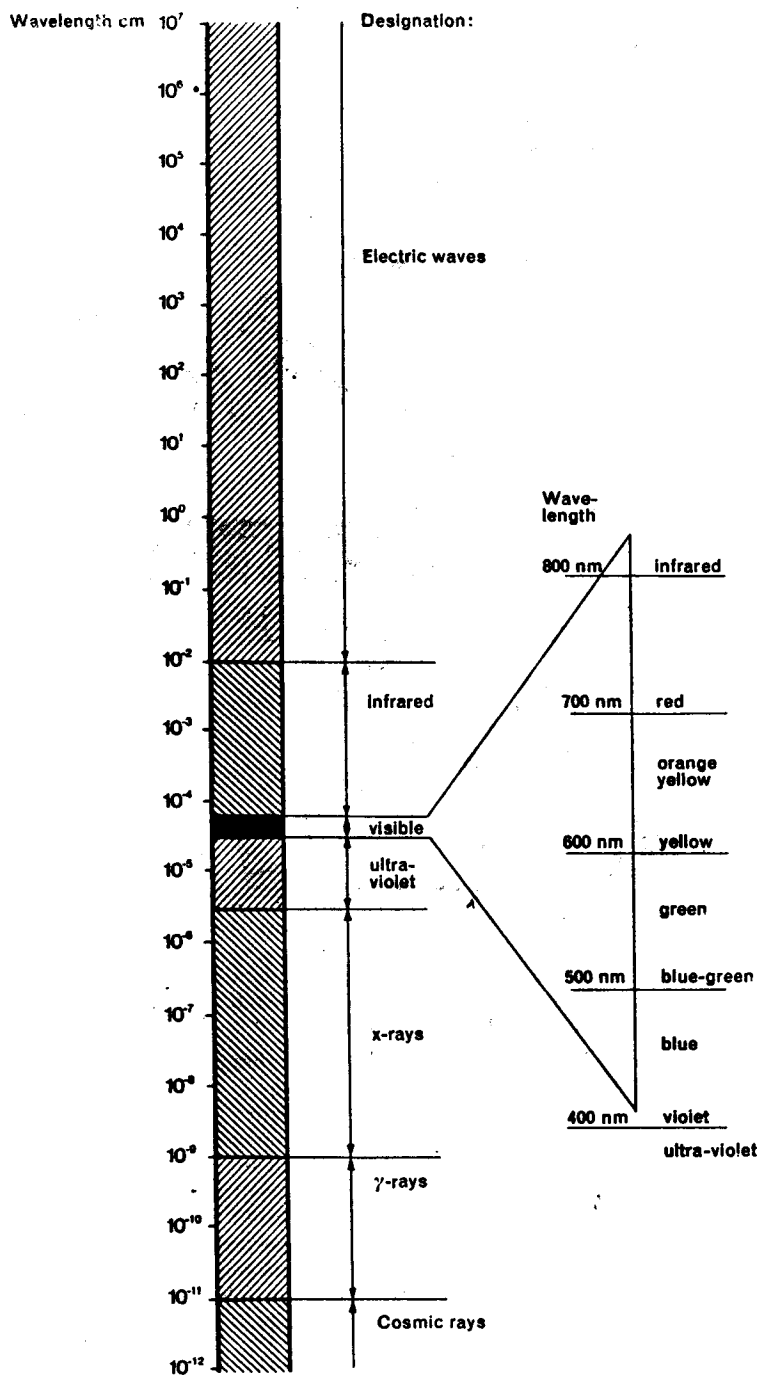


FIG. 2. The electro-magnetic spectrum of radiation (Figure by courtesy of Wild Heerbrugg, Switzerland).

Each wavelength of the electromagnetic spectrum can be associated with a wave number—the reciprocal of the wavelength in cm; and since they are all propagated with the same velocity each will have a different frequency, ν . Each wavelength is also associated with a specific energy E , where $E = h\nu$ is the energy in ergs of a quantum of frequency, ν , where h , is Planck's constant; or the energy value may be expressed as V , the energy in electron volts. In general terms we can say that the shorter the wavelength the higher the energy content and the more penetrating (and dangerous) the radiation; while the longer the wavelength the lower the energy and the less penetrating the radiation. Thus we find a progressive increase of energy from ultraviolet through X- and γ -rays to cosmic rays the most penetrating of all.

A. Theories of light

The earliest theories of light were attempts to explain the phenomenon of vision rather than the nature of light itself. As early as 500 B.C. Greek thinkers had put forward the "tactile" and "emission" theories; the former postulated that the eye sent out invisible sensors or sensitive probes which were able to "feel" objects too distant to be touched physically, while the latter postulated that the object itself emitted something which entered the eye and affected some sensitive part of the eye which was responsible for sight. For reasons obvious to us now, the "emission" theory eventually completely displaced the "tactile" theory, and by giving the emission the term "visible radiation" we can provide a reasonable explanation of the visual process as follows.

Visible radiation emitted (e.g. on heating), reflected or scattered by a body, on entering the eye, is focused by the eye lens onto the retinal surface which contains special sensitive cells connected by nerves to the brain. When light falls on these a chemical and physical reaction takes place involving the transformation of the pigment visual purple and resulting in the emission of electrical impulses to the centre of the brain where the visual image is "recomposed" and results in the sensation of sight. The physiology of vision will not be discussed here.

Quite apart from any physiological consideration it is possible to show experimentally that visible radiation is associated with the transfer of energy and any theory of light must accommodate the energy phenomena associated with it. By analogy with other known methods for the propagation of energy we could propose that light conveys energy in the form of "waves" (as the sea transports energy through its wave motion), or the energy may be conveyed as discrete quantities associated with the movement of particles (as moving billiard balls, for example, possess kinetic energy). It should be noted that the transfer of energy in waves need not involve

the physical translocation of the medium through which the wave passes, either of water, or, in the case of light, of air. These considerations led to two basic theories of the nature of light: the "wave" theory and the "corpuscular" theory.

From the 17th century it has been known that the propagation of light could be represented by rays and simple experiment showed that those rays travelled in straight lines translating energy along a path from source to receptor. From the study of the interference phenomena associated with "Newton's Rings", Newton recognized that there was some sort of periodicity associated with light which was evidence for a wave theory; on the other hand, he could not reconcile this with the rectilinear propagation of light and, incorporating the concepts of his laws of motion, preferred to explain light as a procession of corpuscles which either possessed an internal vibration of their own or were in some sense controlled by waves or vibrations of the medium through which the light travelled. This objection to a simple wave theory was removed when it was discovered that light is not propagated in a strictly linear fashion, and that the rays of a beam of light which impinged on the edge of an object were bent away from the direction of propagation—the phenomenon known as *diffraction*.

As a result of many experiments on interference and diffraction a set of determinations of the wavelength of light were made and it was shown conclusively that different wavelengths were always associated with different spectral colours. The wavelengths of the different spectral colours are shown in Fig. 2.

While the simple wave theory could be used to describe the behaviour of light under many circumstances there were many inadequacies of the theory which were resolved when Maxwell formulated the equations of electromagnetism and showed how these could be used to describe the behaviour of light. An essential feature of the theory was the propagation of transverse electromagnetic waves (a vector quantity) as distinct from the simple theory of longitudinal waves in which the direction of vibration is always the same as the direction of propagation, so that longitudinal wave motion could be represented as variations of a scalar quantity.

The work of Maxwell soon led to the enormous expansion of the spectrum and the realization that "light waves" were only a tiny section of a very much larger spectrum of electromagnetic radiation associated with wavelengths from over 3000 m to less than 10^{-13} m.

Even Maxwell's electromagnetic explanation of the nature of light was inadequate to explain certain phenomena such as the energy transferred to the electron of an atom which had been excited by radiation with subsequent ejection of the electron (ionization). The failure resulted from the implication that the energy of electromagnetic radiation was continuously

distributed. This difficulty was surmounted by Einstein's explanation that the energy of the radiation was concentrated into separate discrete packets, each packet being called a photon, and that for any particular wavelength all the photons had the same energy value. Coupled with Plank's realization that the energy is emitted in multiples of a single unit, with no fractions of a unit being possible, the unit of energy was called the quantum and the relationships between the energy level of a radiation and the wavelength were given by—

$$E = hc/\lambda$$

Where h is Plank's constant (6.6×10^{-34} joule sec), c is the velocity of light and λ the wavelength.

If ν is the frequency then,

$$c = \nu\lambda$$

and

$$E = h\nu$$

The modern theory of light is a composite theory which incorporates both the electromagnetic (wave) theory (which has no place for photons) and the photon (particle or corpuscle) theory which has no place for the waves. The former describes adequately the phenomena of interference, diffraction and polarization while the latter is required for an explanation of the observed interactions of radiation and matter.

Modern quantum mechanics constitutes a single theory incorporating the appropriate parts of the electromagnetic wave theory, the quantum theory and the theory of relativity in a composite explanation of the properties of light and matter.

For an elementary understanding of the behaviour of light in relation to microscopy we can disregard the quantum theory. The quantum aspects are of importance, however, in understanding the phenomenon of fluorescence which lies at the heart of the fluorescence microscopy techniques which are now used to such great effect. Fluorescence microscopy is dealt with elsewhere in this Volume (Walker *et al.*, this Volume, page 219).

B. Light waves

For our present purposes we may depict light as consisting of waves such as that represented by the sine curve shown in Fig. 3.

This form of simple harmonic motion is describable by the trigonometric function—

$$y = A \cos 2\pi \left(\frac{x}{\lambda} - \frac{t}{T} \right)$$