METHODS IN MEDICAL RESEARCH

Volume 3

R. W. GERARD, Editor-in-Chief

METHODS IN
Medical Research

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RALPH W. GERARD, *Editor-in-Chief*

GENETICS OF MICRO-ORGANISMS, *S. E. Luria, Editor*

ASSAY OF NEUROHUMORS, *I. H. Gaddum, Editor*

SELECTED PSYCHOMOTOR MEASUREMENT METHODS,
Walter R. Miles, Editor

METHODS FOR STUDY OF PEPTIDE STRUCTURE, *Choh Hao Li, Editor*

PREFACE

THIS SERIES of volumes is devoted to methods and techniques and there were three main bases for our opinion that such a series might be useful. In the first place, while the results of investigation are constantly subject to critical review, it is less easy to find collected anywhere an appraisal and discussion of the various methods that have been proposed for the solution of some experimental problem. In the second place, it has (especially in physiology) become difficult to secure publication of a paper dealing solely with a technique, or even to include an adequate description of the technique in a paper describing the results obtained. Third, it often happens that a method is modified and improved in continued use, either in the laboratory whence it originated or elsewhere, and such useful modifications find their way into print, if at all, only as brief and scattered indications, and are to a great extent diffused by the uncertain process of personal communication.

Volumes 1 and 2 met with a reception cordial enough to assure us that our confidence in these reasons for launching this series was not misplaced. We hope also that we have learned lessons, and that this volume and its successors will be still more warmly welcomed. The value of the whole series will increase as a widening field is gradually covered.

Each volume is divided into three to five principal, self-contained sections, each representing, for that volume, one of the broad fields of medical research, such as biochemistry, physiology and pharmacology, microbiology and immunology, and biophysics including radiobiology. Within each of these fields we try, year by year, to select narrower topics wherein a restatement of techniques seems timely.

As the topics are selected, we try to find experts, like those who have so signally contributed to the first volumes, willing to act as associate editors for their assigned topic for the year. The responsibilities of the associate editor are by no means light; it is for him to select, within the topic and space assigned, the methods most worthy of description and the contributors best fitted to describe. Obviously the methods most suitable for description in this form are those which are of wide actual or potential application and which have not been published in full or have been usefully modi-

fied since publication; obviously, too, the inclusion of a method stamps it as being convenient and reliable in the associate editor's expert estimation, though it does not conversely follow that omitted methods are of lesser value. The associate editor may also send each contribution to another experienced investigator for comment and review; this feature of our plan has been found most valuable to all concerned.

As members of the Governing Board, we are very conscious of the lightness of our responsibilities in comparison with those of the associate editors and, still more, those of the investigators, who agree to assume the further ungrateful task of acting as editors-in-chief, charged among other things with the duty of distributing space among the sections. Dr. R. W. Gerard, who accepted the responsibility of editorship for this volume, has made it a worthy companion of its predecessors.

Any values which this series may have must be credited to the editors, the contributors and the referees, rather than to us. It remains for us merely to select topics and to try to find equally competent and conscientious editors for the next volume and its successors. To this end we should most gratefully receive any suggestions that readers may care to send us. Volume 4 is in active preparation and will be available in the winter of 1950–51.

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EDITOR'S PREFACE

THERE is little for the editor-in-chief to say in introducing this volume, as there was little for him to do in preparing it. The four sections were assigned to the four associate editors at the start and each planned the organization and authorship of his material with a free hand. This properly identifies the editor-in-chief as the fifth wheel—perhaps a bit of a steering wheel to guide the volume from its conception by the Governing Board to its delivery to the publisher; perhaps a bit of a flywheel to keep the other wheels turning through low pressure portions of the cycle. In any event, I do wish to acknowledge the far greater contributions of the associate editors, and of course of the authors, than of myself to Volume 3 of *Methods in Medical Research*. All have been most co-operative and pleasant to work with and some even achieved the almost unbelievable, in making the deadline and with manuscripts not exceeding the space allotment.

The great Ludwig wrote "Die Methode ist alles," and surely the advance of science attests the overwhelming importance of methodology. True, without the creative insights of the few, the cumulative labors of the many would be largely barren. But, conversely, only as ideas are winnowed in the laboratory are the grains of "truth" selected. Man does not yet know how to produce genius or even talent, so little can be done—except to give the able opportunity—to enhance the trickle of major ideas. One idea, suddenly conceived, however, may require years of men-hours of experimentation for its testing and exploitation. And much can be done to facilitate this dominant character of science—the laboratory testing.

Apparatus of ever greater potency, procedures of ever greater delicacy, require ever greater know-how for their successful use. Such operational wisdom can be acquired initially only painfully by the pioneer. Later, it is passed along and improved by his immediate disciples and by others with related experience. Published accounts, in the usual research reports, are notoriously incomplete and scattered; and spatially separated workers rarely can find, even in the never-fully-adequate written word, the help that would minimize their own expenditure of time in mastering a needed method.

Hence these volumes. In each is gathered detailed operative information for the experimenter in each of several fields. The

articles deal overwhelmingly with methods, described in sufficient detail to eliminate the expensive blunders of ignorance. Much of the detail is empirical, based on the extensive experiences of individuals or laboratories that have pioneered and led in their experimental fields. This know-how has further been checked in many cases by having other experts read and comment on the initial presentations; so that their experience has been incorporated in, or is supplement to, the main articles.

Earlier volumes have been warmly received, presumably because they proved useful. It is the hope of all who have contributed to the present one that it will help their scientific colleagues to greater and easier productivity in their experimentation.

—R. W. GERARD.

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SECTION I

Genetics of Micro-Organisms

ASSOCIATE EDITOR—*S. E. Luria*

INTRODUCTION

IT MAY SEEM surprising that a section on genetics of micro-organisms should appear in *Methods in Medical Research*. The most obvious rationale for this inclusion is the importance of variation in the characteristics of the parasites for the pathology and epidemiology of infectious diseases. Other more subtle though at least as cogent reasons justify the inclusion. On the one hand, all those familiar with the recent advances in biochemical research realize the role that the biochemical genetics of micro-organisms has played in the tracing of synthetic metabolic pathways and in studies on intermediary metabolism. The well substantiated postulate of comparative biochemistry concerning the fundamental similarity of biochemical processes throughout the biologic world justifies the increased use of micro-organisms as models in research on intermediary metabolism. On the other hand, the interest of medical scientists in genetics itself, as a key to the clarification of normal and abnormal patterns of function, is increasing at a fast pace, and it is noteworthy that genetic research in medical schools and institutions is limited as yet, with few exceptions, to the 2 fields of human and of microbial genetics.

The choice of topics to be included in the section was guided by a consideration of the availability, for certain groups of organisms, of methods specifically devised for genetic studies and applicable, with more or less obvious modifications, to the study

of other organisms or of other problems. The 4 chapters include methods in genetics of bacteria, yeasts and molds. Viruses are not included, since the only group that has been investigated intensively from a genetic standpoint—that of bacterial viruses or bacteriophages—is discussed in Volume 2 of this series.

It was hoped that a chapter on the genetics of Paramecium could be included, particularly since many methods used in this most important field had not yet been published. On request of the editor, Dr. T. M. Sonneborn kindly undertook the preparation of such a paper, but the very extent to which this field has grown caused the manuscript to be too long for inclusion in this volume. This important paper appears elsewhere (6).

For bacteria, the methods discussed cover 2 topics: the mutations affecting resistance to destructive agents, and the mutations recognizable by nutritional studies (commonly, if improperly, called "nutritional" or "biochemical" mutations). These are the 2 groups of phenomena that have been studied by methods developed with the specific purpose of investigating the genetic system of bacteria. Genetic research on problems such as bacterial dissociation and antigenic variation is still in such a fragmentary stage that a standardization of methods would be not only unjustified but possibly harmful to much needed further progress. A discussion of methods used in the study of induced "type transformations" in Pneumococcus and other bacteria was also considered unnecessary, since the original observations are still under investigation and their empiric methodology is in the process of being rationalized.

The impact of the recent developments in bacterial genetics on bacteriologic research has been remarkable, as may be judged from the number of articles dealing with genetic problems that have appeared in bacteriologic publications since 1944 and from the prominence accorded genetic subjects at recent annual meetings of the Society of American Bacteriologists. The peculiar methodologic problems involved in bacterial genetics (2, 4), however, are not always understood clearly by those bacteriologists who direct their attention to genetic questions. One of the most frequent pitfalls, particularly in the distinction of spontaneous from directly induced mutation, is the overlooking of the complexities of selective forces in the continually changing environment of the cultural media, complexities that can only be elucidated in each specific instance by careful population studies. No methodology can yet be recommended for the study of bacterial populations beyond the suggestions made by Witkin in her chapter of this section, but the interested worker is referred to

papers by Braun (1) and Ryan (5) for the analysis of individual complex cases.

The genetics of yeasts, molds and protozoa is less concerned today with the problems of Lamarckism vs. Mendelism than the genetics of bacteria, thanks to the occurrence in the former groups of organisms of well investigated sexual processes. The field is, however, actively progressing both in relation to the problem of the organization of the genetic material, particularly in connection with cytoplasmic inheritance, and in relation to physiologic genetics.

The chapter on yeast genetics presented an opportunity to make available the methods used by 1 of the 2 most productive schools in this field, the methods devised by the other school being discussed in a monograph by its chief exponent (3).

The genetics of molds is represented by a discussion of methods in the genetics of the ascomycete Neurospora, a genus that already ranges with Drosophila and maize among the classic materials of genetic investigation. The successful application of Neurospora mutants to bioassay makes the genetic methods, particularly those for the isolation of new mutants requiring given metabolites, tools of practical value in the hands of all biochemists. The same should be said for the methods described by Lederberg in his chapter on biochemical mutants of bacteria. No other fungi (except possibly certain plant pathogens) have been subjected to genetic investigation to any extent that would justify standardization of techniques.

Cytologic techniques are discussed in connection with Neurospora, since here the cytologic picture has already been integrated solidly with the genetic data.

It has not proved feasible to append to each chapter the comments of a referee. All chapters were read critically by at least 1 expert, whose suggestions were often incorporated into the text by the author. The names of the reviewers are given with each chapter.

The editor gratefully acknowledges the unusual and somewhat unexpected co-operation of the authors in meeting the deadlines for submitting their invited contributions, a co-operation that has made the task of the associate editor an altogether pleasant one.

—S. E. Luria.

REFERENCES

1. Braun, W.: Bacterial dissociation, Bact. Rev. 11:75, June, 1947.
2. Delbrück, M.: Spontaneous mutations of bacteria, Ann. Missouri Bot. Gardens 32:223, April, 1945.

3. Lindegren, C. C.: *The Yeast Cell, Its Genetics and Cytology* (St. Louis: Educational Publishers, 1949).
4. Luria, S. E., and Delbrück, M.: Mutations of bacteria from virus sensitivity to virus resistance, Genetics 28:491, November, 1943.
5. Ryan, F. J., and Schneider, L. K.: The consequences of mutation during the growth of biochemical mutants of *Escherichia coli*, J. Bact. 58:201, August, 1949.
6. Sonneborn, T. M.: Methods in the general biology and genetics of *Paramecium aurelia*, J. Exper. Zool. 113:87, February, 1950.

Isolation and Characterization of Biochemical Mutants of Bacteria

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ALTHOUGH MOST mutations may be ultimately referable to biochemical changes, the term "biochemical mutant" generally refers to a mutant detected by its effects on the nutrition or on a specifically recognizable enzymatic process of the organism (1).

The use of mutagens to increase the proportion of mutants is discussed by Witkin (pp. 23 ff.). The methods presented here aim at separating mutant cells from the nonmutants which usually outnumber them. The techniques have been applied especially to *Escherichia coli,* among bacteria, but have also been used with other organisms.

Media.—The recognition of a nutritional mutant depends on a comparison of its growth on "minimal" and "complete" medium. These terms are relative to the purposes of the investigation. A minimal medium consists only of components essential for the

TABLE 1.—MEDIA FOR *Escherichia coli**

A. MINIMAL MEDIUM† (B. D. Davis)	
Glucose	1
K_2HPO_4	7
KH_2PO_4	2
Na_3 citrate$\cdot 5H_2O$	0.5
$MgSO_4 \cdot 7H_2O$	0.1
$(NH_4)_2SO_4$	1

B. COMPLETE MEDIUM	
Casein digest (N Z Case)	10
Yeast extract	5
K_2HPO_4	3
KH_2PO_4	1
Glucose	5

C. MINIMAL EMB MEDIUM ("EMS") (20)	
Sugar	10
Sodium succinate	5
NaCl	1
$MgSO_4$	1
K_2HPO_4	2
$(NH_4)_2SO_4$	5
Eosin Y	0.4
Methylene blue	0.065

D. COMPLETE EMB MEDIUM	
Sugar	10
Casein digest (N Z Case)	8
Yeast extract	1
NaCl	5
K_2HPO_4	2
Eosin Y	0.4
Methylene blue	0.065

*Agar is always used at a final concentration of 1.5% if required. All concentrations are g/l of distilled water. The media may be prepared by adding the materials and autoclaving together. However, minimal medium A is better prepared by sterilizing the sugar (and agar) in a separate aliquot and mixing with the sterilized solution of the other constituents just before pouring agar plates.

†Trace elements have been dispensed with, being present in adequate amounts in other chemicals of ordinary chemical purity.

growth of the wild-type strain; a complete medium contains a variety of supplements covering the range of interest of growth factors for which mutants are to be sought. Yeast extract, peptones and similar organic preparations are commonly used as complex supplements which are expected to contain adequate amounts of such growth factors. When specific mutants are being looked for, however, it is important to verify that the "complete" medium is sufficiently enriched in the particular factor and that it does not contain antagonists which might interfere with the expected responses (23).

Media suitable for *E. coli* are tabulated in Table 1. They are certainly not the only formulae that will prove satisfactory; for specific purposes it will always be necessary to formulate the appropriate substitutes.

RANDOM ISOLATION OF NUTRITIONAL MUTANTS (2, 8)

PROCEDURE

Cells grown from an irradiated inoculum in a complete medium are diluted to 100–500/sample and poured into or spread on the surface of complete agar plates. After 24 hr of incubation, colonies are picked at random with a fine platinum needle. It is convenient to cool the needle in a small tube containing 1 ml of synthetic medium, touch the colony lightly, immerse it in the 1 ml tube, and then spot the needle on a complete agar plate. One can also use a 2d tube containing complete medium for maintaining the isolate. After 24 hr the tubes or spots on complete agar corresponding to clear minimal tubes (no growth) are transferred to slants for later verification as mutants. Where a spot shows no growth, there may not have been successful inoculation into the minimal medium, and the test is discarded.

Evaluation.—Tatum (35) recovered about 1% characterizable mutants from *E. coli* treated with x-rays or nitrogen mustard. Roepke and Mercer (29) obtained yields of 1–2% from cells plated immediately after x-ray treatment. They noted that storage of treated cells in electrolyte-containing medium resulted in a markedly lower yield. In *Bacillus subtilis,* Burkholder and Giles (4) obtained 3–6% yields of mutants, a high yield which may be related to the resistance of their material to lethal effects of radiation. Substantially the same procedure has been used for Neurospora, where Beadle and Tatum (2) report the isolation of 380 mutants from 68,000 tested ascospores, or about ½%. However, the genetic procedure used in Neurospora is only 50%

efficient because the ascospores are the progeny of a cross of treated with untreated nuclei.

DELAYED ENRICHMENT (LAYER PLATING) (21)

Much of the work needed to obtain a collection of mutants is spent on separating them from their nonmutant neighbors, which often predominate 100:1. The most direct but least efficient procedure is random isolation followed by individual test on minimal medium. By delayed enrichment of minimal agar plates, several hundred colonies can be screened at once.

PROCEDURE

Plates are prepared with 15–20 ml of minimal agar medium and allowed to solidify (bottom layer). The bacterial suspension is diluted to contain 200–400 cells/sample; each sample is incorporated in a few ml of minimal agar and poured over a plate (seeded layer). When this has hardened, 5–10 ml of minimal agar is poured on top (cover layer). Thus all the colonies will develop in the agar and will not be disturbed by subsequent manipulation. The seeded plate is incubated for 24 hr or longer to permit the development of prototroph cells (i.e., cells as nutritionally competent as the wild type) into uniform large colonies. The mutant cells will have developed very slightly, if at all, and will be invisible to the naked eye. They are brought to view by pouring a few ml of complete agar over the layer plates (supplement layer). The growth factors diffuse down through the agar and cause growth of the mutants, which are picked up as small or as new colonies after 6–12 hr of further incubation. With some practice and use of a fine needle, the tiny mutant colonies can be picked to complete medium for verification.

Modifications.—Other methods of delayed enrichment suggest themselves. The supplement need not be poured on top, but may be injected below the bottom layer with a hypodermic needle through a penicillin cup, or placed in a penicillin cup itself, or may be poured in a trench cut out of the minimal agar either with a sterile spatula or by pulling up a strip of cloth sterilized with the plates. These and other modifications, e.g., transfer of the entire agar layer to a plate of complete agar, may be particularly useful in dealing with aerophilic bacteria or molds which will not develop readily as deep colonies.

MARGINAL OR LIMITING ENRICHMENT (6)

A marginal or limiting concentration of growth factor can usually be found at which a mutant will form colonies character-