MANUAL

OF METHODS FOR

PURE CULTURE STUDY OF BACTERIA

OCIETY OF AMERICAN BACTERIOLOGISTS

FT240

This MANUAL is published in loose-leaf form so that it can be revised, leaflet by leaflet, and thus kept up to date. The revised leaflets are issued in the semi-annual publication, PURE CULTURE STUDY OF BACTERIA; each leaflet thus issued is punched to fit the cover of this MANUAL. The subscription rate of PURE CULTURE STUDY OF BACTERIA is $1.25 per year ($1.00 cash).

To keep this copy of the MANUAL up to date, detach the card below and mail it, with remittance to BIOTECH PUBLICATIONS, LOCK BOX 269, GENEVA, NEW YORK.

.....

No. copies subscribed for.....

Amount enclosed.....

Date.....

BIOTECH PUBLICATIONS
LOCK BOX 269, GENEVA, N. Y.

Please enter the above name as a subscriber for PURE CULTURE STUDY OF BACTERIA. The sum of $1.00 is enclosed* for each copy of the MANUAL which it is desired to keep up to date. The number of copies for which subscription is entered is indicated in the right-hand corner above.

*When credit is asked and an invoice issued, the rate is $1.25 a year. In case of an institutional subscription this card should be sent with the official order. *Subscriptions should not be placed thru magazine agencies unless absolutely necessary.*

WFH 162469

MANUAL

OF METHODS FOR

PURE CULTURE STUDY OF BACTERIA

EDITED BY

THE

COMMITTEE ON BACTERIOLOGICAL TECHNIC

OF THE

SOCIETY OF AMERICAN BACTERIOLOGISTS

GENEVA, N. Y.

PUBLISHED BY THE BIOTECH PUBLICATIONS

TABLE OF CONTENTS*

(May 1949)

Leaflet I. Introductory

Leaflet II. Preparation of Media

Leaflet III The Study of Obligately Anaerobic Bacteria

Leaflet IV. Staining Methods

Leaflet V. Routine Tests for the Descriptive Chart

*In these page numbers, the Roman numerals refer to the leaflet, the small inferior numerals to the year of the edition, and the large arabic numerals to the page of the leaflet.

Leaflet VI. Further Biochemical Methods

Leaflet VII. The Study of Pathogenic Aerobes.—Determination of Pathogenicity

Leaflet VIII. Serological Methods

Leaflet IX. The Measurement of pH, Titratable Acidity, and Oxidation-Reduction Potentials

Leaflet X. Inoculations with Bacteria Causing Plant Disease

Index

LEAFLET I

INTRODUCTORY

9th EDITION
Pure Culture Study of Bacteria, Vol. 12, No. 1
FEBRUARY, 1944
REVISED, OCTOBER, 1948

LEAFLET I. INTRODUCTORY

PURPOSE OF THE MANUAL

This MANUAL is intended for use in that type of bacteriological work known as "pure culture study of bacteria", the meaning of which is discussed below.

The methods given here are not to be regarded as official. The committee has always taken the stand that official methods should not be adopted in the case of research work, because it is continually necessary to modify research methods in order to keep them up to date. The standardization of methods tends to hinder the development of new technic, while the chief function of this committee is to stimulate its development. This contention of the committee seems now to be officially recognized by the Society of American Bacteriologists, and this organization has of recent years left the establishment of official methods to other bodies having closer connection with regulatory work. At the request of the Society this Manual now bears upon its title page the statement: "*The methods given have not been formally approved by the Society, and are in no sense* OFFICIAL *or* STANDARD."

The methods in this Manual, therefore, are merely claimed to be the best that have come to the attention of the committee at the time of publication. Whenever practical, the methods have been tested by the committee in comparison with other procedures; when this has not been done, methods are given with a statement to indicate that they have not been critically tested.

MEANING OF PURE CULTURE STUDY

There has sometimes been misunderstanding as to the sense in which the Committee uses the expression "pure culture study of bacteria". It is occasionally thought that such an expression would cover nearly the whole field of bacteriological technic. On the other hand, the definition of pure culture study of bacteria which has been drawn up by the Committee on Bacteriological Technic is: the study of bacterial cultures with the object of learning their characteristics and behavior or determining their identity, or both. Such a study may be regarded as including: isolation methods; methods for the cultivation and the storage of various kinds of bacteria; the microscopic study of pure cultures either stained or unstained; determination of cultural characteristics of an organism; a study of its physiological characteristics; the chemical methods necessary in making the last-mentioned study; the determination of pathogenicity and study

of pathological effects; the serological requirements of an organism when used as a means of characterization.

It is clear from such a statement that Pure Culture Study of Bacteria is fairly comprehensive, but that there are many fields of bacteriological technic not included within it, e.g.: methods for the enumeration of bacteria in their natural habitats; the diagnosis of disease, and many other phases of pathological bacteriology; methods employed in the study of food spoilage and controlling the processes of fermentation, etc. Such a list might be extended almost indefinitely; for the field of pure culture study, although fairly broad, is actually merely a small part of bacteriological technic.

Relation to Taxonomy

Clearly, one of the main objects of pure culture study is to determine the identity of any bacterial culture under investigation. This brings the subject very close to the field of bacterial taxonomy—i.e., the naming and classifying of bacteria. Inasmuch as bacteria cannot be classified without studying their characteristics in pure culture, it is an obvious conclusion that pure culture study is a necessary prelude to bacterial taxonomy.

It must be recognized, nevertheless, that one can consider pure culture study without regard to taxonomy and that one can study the taxonomy of bacteria without paying special attention to the methods of pure culture study. Since this distinction can be made and the committee editing this series of publications is a Committee on *Technic*, care has always been taken to maintain the distinction so as not to interfere with the functions of other committees that have been appointed to deal with matters of nomenclature and classification. It should be remarked, however, that this distinction was not always observed in the past, as a result of which the original committee, from which the present Committee on Technic has descended, was called the Committee on the Identification of Bacterial Species. Thus considered, it was really a committee on one phase of taxonomy. Early in its history, however, it began centering its interests on the technic involved, and about twenty years ago it seemed wise to change its name to the Committee on Bacteriological Technic.

Publications of the Committee on Technic

Descriptive Charts: The first descriptive chart actually adopted by the Society of American Bacteriologists was in 1907. The history of these early developments is given in Leaflet I of the Manual of Methods for Pure Culture Study of Bacteria and hardly needs

to be discussed here. The chart has been revised from time to time and at present there are two forms—one known as the Standard Descriptive Chart, and the other as the Descriptive Chart for Instruction. The latter is very much simpler than the former. The former is printed on both sides of a 8½″ x 11″ sheet of light cardboard, the latter on a sheet of heavy paper of the same size.

The object of the Descriptive Chart is to provide a space for recording the most important characteristics of a single culture. The Standard Chart is the most complete and is intended especially for advanced work in bacteriology. Unfortunately, however, it does not meet modern research needs at all perfectly because each group of bacteria requires its own set of tests and no form can be drawn up sufficiently detailed to cover all of them. The Chart for Instruction, on the other hand, is so much simpler and contains so much blank space that it sometimes is found to be more satisfactory in research work than the Standard Chart. It is, however, intended primarily for students to use in characterizing cultures furnished them in connection with their class work.

Manual of Methods for Pure Culture Study: The origin of this Manual traces back to a Committee report which was printed in the Journal of Bacteriology in 1918 and was distributed in reprint form by the Committee. This report was only 14 pages long and was concerned only with the methods used in carrying out the determinations called for on the Descriptive Chart of those days. The original report was revised once or twice, and in 1923 was finally issued as an independent publication under its present name. The first edition of the Manual was only 48 pages in length. As it was put out in loose-leaf form, however, it was possible to revise it section by section; and each revision has tended to be longer than the preceding. The result is that the present edition contains about 200 pages.

The present Manual consists of ten leaflets and each leaflet has its own pagination. The system of page numbering adopted may seem peculiar and has caused some objections as seeming slightly complicated. It is, however, the simplest form that can be adopted to avoid confusion in a publication of this kind. Serial paging for the entire Manual is impossible because the leaflets vary in size from one edition to the next. As a result serial paging for each separate leaflet has been adopted, and to avoid confusion in page references made elsewhere the number of the leaflet together with the year of publication is given in small figures before the page number itself; thus II_{40}-8 would indicate page 8 of the 1940 edition of Leaflet II, and a reference to it in that form is very exact.

As just stated, the original object of this Manual was to supply the methods to be used in the study of organisms according to the Descriptive Chart. As the subject developed, however, it was felt that there are other fields of pure culture study equally important and these have been added from time to time. The present Manual deals with so many lines of technic that it is essentially a laboratory handbook covering those procedures referred to above as comprising the field of pure culture study. It is coming to be used more and more for this purpose, and in a number of institutions is now put in the hands of all students in certain classes of bacteriology. Thus used, it has the advantage over conventional texts in that the Committee behind it is consistently endeavoring to keep it up to date.

The present edition of this Manual contains ten leaflets bearing the following titles: I. Introductory; II. Preparation of Media; III. The Study of Obligately Anaerobic Bacteria; IV. Staining Procedures; V. Routine Tests for the Descriptive Chart; VI. Further Biochemical Methods; VII. The Study of Pathogenic Aerobes; VIII. Serological Methods; IX. The Determination of pH and Titrable Acidity; X. Inoculations with Bacteria Causing Plant Disease.

The system adopted for keeping the Manual up to date is by means of subscriptions to this quarterly publication, PURE CULTURE STUDY OF BACTERIA. Nearly every issue of this quarterly contains a revision of some one of the ten leaflets. Anyone owning a copy of the Manual can subscribe to PURE CULTURE STUDY OF BACTERIA by filling out the card attached to the front of the Manual and sending it in to the publishing agency with a year's subscription. Thus, any student who first purchases a copy merely in connection with his class work, can easily arrange to have it kept up to date if he finds that he is going into bacteriological work permanently. It is in this way that the owner is able to profit from the loose-leaf type of publication which has been adopted.

HISTORICAL

The first efforts toward producing a descriptive chart for characterizing bacteria were made by two different individual investigators, H. W. Conn, and S. de M. Gage. The work of these two investigators called the matter to the attention of bacteriologists in general and it was finally brought before the Society of American Bacteriologists by F. D. Chester at the Philadelphia meeting in December 1903, and then again at the 1904 meeting when he explained his idea of a "group number" which would be descriptive of the salient characters of an organism. On his recommendation the Society appointed

a Committee on Methods for the Identification of Bacterial Species of which Prof. Chester was made chairman. This committee drew up the first descriptive chart with which the Society of American Bacteriologists had any connection.

This chart was put before the Society at its 1905 meeting. It was presented at this time as a preliminary effort and no endorsement of it was given by the Society nor apparently was such endorsement requested. The committee was instructed to continue its work and a second chart was prepared during 1906 and presented at the Society meeting in December that year. At this meeting it was decided that the chart should call for more complete data concerning bacteria than provided for by either of the two charts already submitted; so the committee was instructed to do further work along this same line.

The committee at this time was composed of F. D. Chester, F. P. Gorham, and E. F. Smith; but Prof. Chester was largely responsible for the first two charts presented at Society meetings. Before the committee undertook a further revision, however, he had left bacteriological work and hence was no longer active on the committee. During 1907, therefore, Dr. Smith acted as chairman of the Committee and under his supervision the committee drew up another chart which was presented to the Society at its meeting in December that year. This chart was officially endorsed by the Society and was put on sale by the secretary of the Society.

For several years following no changes were made in the chart. The next step in its development was brought about by H. A. Harding (1910), who published a paper in which he outlined the complete history of the chart, with copies of the early charts, and discussed improvements that might be made. This paper is available for those desiring more detail concerning this early history than is given here.

As the Society felt that further modifications were now needed a new committee was appointed in 1911 consisting of F. P. Gorham, C. E. A. Winslow, Simon Flexner, H. A. Harding and E. O. Jordan. This committee gave a report at the 1913 meeting, presenting a chart which was put on sale by the Society, but was not officially endorsed. As this committee was unable to continue the work, an entirely new one was appointed at this time, consisting of H. A. Harding, H. J. Conn, Otto Rahn, W. D. Frost and I. J. Kligler. This committee soon lost Dr. Rahn, who left the country in 1914, and M. J. Prucha was added in his place. The committee was called the Committee on Revision of the Chart for the Identification of Bacterial Species.

The new committee was instructed by the Society to make a conservative revision of the chart and at the same time to draw up a manual of methods to be used in connection with it. At the 1914 meeting of the Society, therefore, a chart was presented for approval, much like the 1907 chart except for its more logical arrangement of data. This chart was given the Society's endorsement and was issued during 1915.

The 1914 chart was printed on a sheet with its back entirely blank, the glossary previously on the back having been omitted. The committee gave as the reason for this that the glossary should be included in the manual on methods shortly to be published. The publication of this manual was delayed, however, pending investigation of the methods to be included in it. This investigation of methods was to be undertaken not only for the sake of the manual but also as a preliminary step toward radical revision of the chart, which was felt to be badly needed. Early in 1917, however, and before this program could be carried out, the chairman of the committee was forced by pressure of other duties to drop the work. As he wished to remain on the committee, however, no change in membership was made, but H. J. Conn was asked to become chairman.

The committee then undertook the first step toward the preparation of a manual on methods. A report was presented at the 1917 meeting, giving the methods recommended at that time for use with the chart. The report was printed in the *Journal of Bacteriology*, March 1918, and was subsequently sold by the Society in the form of reprints. This report was considered a preliminary manual on methods.

The committee proposed at the same time a much simplified chart in the form of a four page folder, which it recommended for use in instruction until the official chart could be given the revision it needed. This chart was not endorsed by the Society; but was printed and sold by the Society for two or three years.

This same committee (but now called the Committee on the Descriptive Chart) issued another report on methods which appeared in the *Journal of Bacteriology*, March 1919, dealing with the Gram stain, production of acid, and the reduction of nitrates. At the 1919 meeting it issued a further report which appeared in the *Journal of Bacteriology*, in two parts, March and May, 1920. The first part of the report was a revision of the one which had been published in March 1918, and was sold as a revised manual of methods until the reprints were exhausted in 1922.

At the 1920 meeting the Committee on the Descriptive Chart was discharged with the understanding that its functions would be taken

over by a committee of broader scope then appointed and called the Committee on Bacteriological Technic. This committee was appointed with the understanding that its membership should fluctuate from year to year in order to keep on it men actively interested in the work.

The new committee made a further revision of the chart, which was presented at the 1920 meeting and endorsed by the Society. Later editions of this chart have been drawn up by the committee in the years of 1924 and 1929, but neither of these have been submitted to the Society for official endorsement. In order to avoid committing the Society in favor of any of the methods concerned, recent editions of the Chart have merely been presented by the committee and permission asked to put them on sale.

The committee issued four further reports in the *Journal of Bacteriology*, (1921, 1922 a, b, & c) before this Manual was prepared. One of these reports (1922b) proposed certain revisions of methods, in the case of the Gram stain, fermentation, nitrate reduction, indole and hydrogen sulfide production. The committee presented this report at the 1922 meeting of the Society with the recommendation that the revised material be published as part of a Manual of Methods for Pure Culture Study of Bacteria. The committee was thereupon instructed by the Society to publish this Manual, using the loose-leaf form of binding, with the understanding that new folders be issued from time to time to keep it up to date.

The Committee on Bacteriological Technic has seen the following changes in personnel:

1920 H. J. Conn, K. N. Atkins, I. J. Kligler, J. F. Norton, G. E. Harmon.

1921 H. J. Conn, K. N. Atkins, G. E. Harmon, Frederick Eberson, Alice Evans.

1922 H. J. Conn, K. N. Atkins, G. E. Harmon, Frederick Eberson, F. W. Tanner, and S. A. Waksman.

1923 H. J. Conn, K. N. Atkins, J. H. Brown, G. E. Harmon, G. J. Hucker, F. W. Tanner, and S. A. Waksman.

1924–5 H. J. Conn, K. N. Atkins, J. H. Brown, Barnett Cohen, G. J. Hucker, F. W. Tanner.

1926–7 H. J. Conn, Barnett Cohen, Eliz. F. Genung, W. L. Kulp, W. H. Wright; with G. J. Hucker and S. Bayne-Jones as a sub-committee on serological methods.

1928 H. J. Conn, Victor Burke, Barnett Cohen, Eliz. F. Genung, W. L. Kulp, W. H. Wright.

1929–30 H. J. Conn, Victor Burke, Barnett Cohen, Eliz. F. Genung, I. C. Hall, W. L. Kulp, W. H. Wright (deceased, May, 1929).

1931–4 H. J. Conn, Barnett Cohen, Eliz. F. Genung; Victor Burke (pathological methods); I. C. Hall (anaerobic methods); J. A. Kennedy (serological methods).

1935 H. J. Conn, Victor Burke, Barnett Cohen, W. M. Jennison, J. A. Kennedy.

1936–42 H. J. Conn; J. H. Brown (anaerobic methods) Victor Burke, (pathological methods); Barnett Cohen, C. H. Werkman, (biochemical methods); M. W. Jennison, (the Descriptive Chart); J. A. Kennedy (serological methods); A. J. Riker (plant pathological methods).

1943–5 H. J. Conn, Victor Burke, Barnett Cohen, C. H. Werkman, M. W. Jennison, J. A. Kennedy, L. S. McClung, A. J. Riker.

1946-7 H. J. Conn, G. H. Chapman, Barnett Cohen, I. C. Gunsalus, M. W. Jennison, L. S. McClung, A. J. Riker, C. E. ZoBell.

1948- M. W. Jennison, G. H. Chapman, Barnett Cohen, H. J. Conn, I. C. Gunsalus, J. A. Kennedy, L. S. McClung, A. J. Riker, C. E. ZoBell.

USE OF THE MANUAL

Pitfalls to be Avoided by the Student

In studying bacterial cultures with the object of identifying them or describing them, the student is apt to run onto certain pitfalls. Many of these are well known and others less fully appreciated. At the risk of making comments that are already too well known by students of bacteriology, a few words concerning some of these pitfalls do seem called for here. They arise primarily from three sources: first, the danger of impure cultures; second, confusing results due to variation of bacterial species; third, differences in methods of study.

The danger in impure cultures is, of course, thoroughly understood. Unfortunately, however, the second consideration just mentioned makes it more important to emphasize the danger of impure cultures today than was the case 25–30 years ago. In those days bacteriologists quite generally accepted the idea of monomorphism; and whenever a culture was observed to be noticeably abnormal either in morphology or physiology, it was promptly discarded as a contaminant. When, however, it began to be learned that even the most strictly guarded pure cultures might show changes in morphology during their life history, and then later when it was realized that the same organism might occur in two or more phases showing distinctly different cultural and physiological characteristics, the old ideas of monomorphism were decidedly upset. As a result of the changing point of view, it is very easy for a careless student today to believe that he is observing two phases of the same pure culture when actually one of his "phases" is a contaminant. This makes constant checking as to purity of cultures even more important than it was before dissociation into phase variants was generally accepted by bacteriologists.

Accepting the idea of dissociation presents other difficulties to the student. Without exhaustive study, it is sometimes very easy to describe two phases of the same species as though they were different organisms. It is also easy to prepare a description of some culture which is an illogical jumble of the characteristics of two or more

phases, due to the fact that it was first studied in an unstable form and dissociation was taking place during the course of the study. On the other hand, some of the methods employed in the hopes of inducing phase variation may actually cause contamination and be incorrectly interpreted. Some of these points are very adequately discussed by Frobisher (1933).

The third source of error above mentioned (variation in methods) also needs emphasis. When a species is described in such terms as one frequently encounters in published descriptions—e.g. "Produces acid (without gas) from glucose and lactose but not from sucrose; does not reduce nitrates"—one has to guess at the answers to such questions as these: What basal medium was used in each instance? What indicator of acid production was employed? How thorough a study was made to show the absence of any acid from sucrose, or of any reduction of nitrate? Or, in the latter instance, is it safe to assume that the author of the species merely failed to find nitrite in some nitrate medium? Unless such questions are answered correctly, the description is meaningless, the attempt to identify an unknown culture with such a description may well give misleading results.

With all these pitfalls to avoid, it is easy to see how the same set of data, no matter how carefully prepared, can be differently interpreted by two different bacteriologists. As a result extreme caution is urged, both in determining the identity of a culture and in deciding whether or not to pronounce it a new species.

Practical Hints

Determining the characteristics of a culture: One should always, if possible, make a complete study of a culture promptly after its first isolation while it is in vigorous condition. When a culture has become attenuated in the laboratory, it should be restored to vigor by growth under conditions well suited for its invigoration. When this is done, however, the possibility should always be recognized that by such "invigoration" dissociation may be induced so that the phase subsequently studied may be quite different from the original isolation. Whenever distinct evidence of dissociation is observed, each phase should be studied and recorded separately; and efforts should be made to reverse the change or to obtain the same change with other strains until the possibility of impure cultures seems to be out of the question. No importance should ever be attached to a single determination, unless supported by a duplicate or even by triplicates giving the same results. In describing morphology, one

should not be contented with one or two observations, but should study several transfers and should follow up each of them day by day for about a week. When changes are observed, a careful study should be made to learn whether they indicate morphologic variation, dissociation, or merely contamination. In making special staining tests, like the Gram stain, several determinations should be made on separate transfers of the culture and at different ages, because there are species that vary in their staining reactions, and such variation cannot be detected by single determinations. As a check on the technic, a known positive and a known negative culture should be included in the study. For example, when making a Gram stain, it is good practice to place on the slide, beside the culture under study, a smear containing a mixture of a known Gram-positive and a known Gram-negative organism (which differ markedly in morphology). Then it is possible to observe whether the expected results are obtained with the known cultures, and thus to have some degree of control on the technic.

Identification: After recording the characteristics of an organism, the next step is identification, if possible, with a previously described species. This should never be attempted until at least six representative strains of the unknown organism isolated from more than one source, if possible, have been studied. No rules can be given for identifying the culture. Descriptions of bacteria are scattered so widely through the literature and vary so greatly in their form that identification is often extremely different. Bergey's Manual of Determinative Bacteriology is a great help; but it is usually necessary to go back to original descriptions and often to secure transfers of authentic strains before certain identification can be made. Difficult as this procedure is, no one is justified in naming a new species of bacteria until a comprehensive search through the literature of species already described has been made. Frequently it is necessary to refer in some publication to a previously described species on the basis of such an identification as this. In this case it is important to state in the publication whether or not an authentic strain of the species has been obtained for comparison; if so, from where obtained; if not, what published description of the species was followed in making the identification. As to a name to use for such a species one may follow the original author's nomenclature or may give it the name employed in some modern system (e.g. Bergey). Whatever name is chosen no confusion will result it if is accompanied by the name of the original author of the specific name and by that of the one making the combination of generic and specific names. Thus,

whether one says "*Bacillus coli* Migula" or "*Escherichia coli* (Migula) Castellani and Chalmers", it is entirely clear what species is intended.

Naming a new species: When it proves impossible to identify a culture with any species described in the literature, it is often desirable to publish a description of it as a new species. When publishing such a description, there are five important points to be kept in mind: (1) The description should be based on at least six representative isolations of the organism. (2) If variations are found to occur among these strains, a critical study must be made to be sure that they are not the result of contamination. (3) In naming any characteristic of the species, especially if it is a negative character (e.g. "nitrates not reduced"), the technic by which it is determined must be stated. (4) Before giving the results of any test as positive or negative, comparisons must be made with a control culture known to be positive and one known to be negative. (5) Before actually assigning a name one should consult a specialist in bacterial taxonomy, both as to the necessity for a new name and as to the validity of the name selected. The Board of Editor-Trustees of Bergey's Manual, for example, are always very glad to offer such advice.

If these hints were followed by all who are trying to identify species or to publish descriptions of them, much of the confusion in bacterial nomenclature would be eliminated.

REFERENCES

HARDING, H. A. 1910. The constancy of certain physiological characters in the classification of bacteria. N. Y. Agric. Exp. Sta. Tech. Bul. 13.

COMMITTEE ON DESCRIPTIVE CHART. 1918. Methods of Pure Culture Study. Jour. Bact., **3**, 115–128.

COMMITTEE ON DESCRIPTIVE CHART. 1919. Methods of Pure Culture Study. Progress report for 1918. J. Bact. **4**, 107–132.

COMMITTEE ON DESCRIPTIVE CHART. 1920 a. Methods of Pure Culture Study. Revised. J. Bact. **5**, 127–143.

COMMITTEE ON DESCRIPTIVE CHART. 1920 b. Progress report for 1919. J. Bact. **5**, 315–319.

COMMITTEE ON BACTERIOLOGICAL TECHNIC. 1921. Progress report for 1920. J. Bact. **6**, 135–141.

COMMITTEE ON BACTERIOLOGICAL TECHNIC. 1922 a. An investigation of American Stains. J. Bact. **7**, 127–248.

COMMITTEE ON BACTERIOLOGICAL TECHNIC. 1922 b. Methods of Pure Culture Study. J. Bact. **7**, 519–528.

COMMITTEE ON BACTERIOLOGICAL TECHNIC. 1922 c. An investigation of American gentian violets. J. Bact. **7**, 529–536.

FROBISHER, M. 1933. Some pitfalls in bacteriology. J. Bact. **25**, 565–71.

GLOSSARY OF TERMS USED IN THE MANUAL AND ON THE DESCRIPTIVE CHART

Acid curd, coagulation of milk due to acid production.

Adherent, applied to sporangium wall, indicates that remnants of sporangium remain attached to endospore for some time.

Aerobic, growing in the presence of free oxygen, **strictly aerobic** growing only in the presence of free oxygen.

Agglutinin, an antibody having the power of clumping suspensions of bacteria.

Anaerobic, growing in the absence of free oxygen; **strictly anaerobic,** growing only in the absence of free oxygen; **facultative anaerobic,** growing in both presence and in absence of oxygen.

Antibody, a specific substance produced by an animal in response to the introduction of an antigen.

Antigen, a substance which when introduced into an animal body, stimulates the animal to produce specific bodies that react or unite with the substance introduced.

Antigenic action, behavior as an antigen.

Antitoxin, an antibody having the power of uniting with or destroying a toxic substance.

Arborescent, branched, tree-like growth.

Aseptically, without permitting microbial contamination.

Autotrophic, able to grow in absence of organic matter.

Bacteriocidal, capable of killing bacteria.

Bacteriostasis, preventing bacterial growth, but without killing the bacteria.

Beaded, (in stab or stroke culture) separate or semi-confluent colonies along the line of inoculation.

Bipolar, at both poles or ends of the bacterial cell.

Bleb, vesicle or blister-like swelling.

Brittle, growth dry, friable under the platinum needle.

Butyrous, growth of butter-like consistency.

Capsule, an envelope surrounding the cell membrane of some kinds of bacteria.

Chains, four or more bacterial cells attached end to end.

Chromogenesis, the production of color.

Clavate, club-shaped.

Compact, refers to sediment in the form of single fairly tenacious mass.

Complement, a non-specific enzyme-like substance, destroyed if subjected to heat (56°C or over for 30 minutes), which occurs in blood serum, and is necessary, in conjunction with a specific antibody, in order to bring about cytolysis.

Concentrically ringed, marked with rings, one inside the other.

Contoured, an irregular, smoothly undulating surface, like that of a relief map.

Crateriform, a saucer-shaped liquefaction of the medium.

Cuneate, wedge-shaped.

Curled, composed of parallel chains in wavy strands, as in anthrax colonies.

Cytolysin, an antibody causing cytolysis.

Cytolysis, a dissolving action on cells.

Diastatic action, conversion of starch into simpler carbohydrates, such as dextrins or sugars, by means of diastase.

Diphtheritic, diphtheria-like.

Dissociation, separation of characters, usually referring to phase variation (q. v.).
Echinulate, a growth along line of inoculation with toothed or pointed margins.
Edema, intercellular accumulation of fluid in a part of an animal body.
Effuse, growth thin, veily, unusually spreading.
Endospores, thick-walled spores formed within the bacteria; i. e., typical bacterial spores like those of *B. anthracis* or *B. subtilis.*
Endotoxin, a toxic substance produced within a microorganism and not excreted.
Enzyme, a chemical ferment produced by living cells.
Erose, irregularly notched.
Excentric, slightly to one side of the center, between the positions denoted central and subterminal.
Exogenous, originating outside the organism.
Exotoxin, a toxic substance excreted by a microorganism and hence found outside the cell body.
Facultative anaerobe, see *anaerobic.*
Filamentous, growth composed of long, irregularly placed or interwoven threads.
Filaments, applied to morphology of bacteria, refers to thread-like forms, generally unsegmented; if segmented, the organisms are enclosed in a sheath.
Filiform, in stroke or stab cultures, a uniform growth along line of inoculation.
Flagellum (*pl.*-la), a motile, whip-like attachment; an organ of locomotion.
Flaky, refers to sediment in the form of numerous separate flakes.
Flocculent, containing small adherent masses of various shapes floating in the fluid.
Fluorescent, having one color by transmitted light and another by reflected light.
Gonidia, asexual spores.
Gonidial, referring specifically to a bacterial phase producing gonidia-like bodies.
Granular, composed of small granules.
Hemolysin, a substance causing hemolysis either alone or in presence of complement.
Hemolysis, a dissolving action on red blood corpuscles.
Hemorrhage, an escape of blood from the vessels.
Histolysis, breaking down of tissues.
Hydrolysis of starch, destruction of starch by the formation of a chemical union with water; includes diastatic action, but is a more general term.
Immune serum, an animal fluid containing an antibody.
Inactivate, to destroy complement by heat (at 56° for 30 minutes).
Infundibuliform, in form of a funnel or inverted cone.
Intraperotoneal, within the peritoneum.
Intravenous, within a vein.
Iridescent, exhibiting changing rainbow colors in reflected light.
Lesion, a local injury or morbid structural change.
Lobate, having lobes, or rounded projections.
Maximum temperature, temperature above which growth does not take place.
Membranous, growth thin, coherent, like a membrane.
Metabolite, a substance produced by metabolism.
Microaerophilic, growing best in presence of small quantities of oxygen.
Minimum temperature, temperature below which growth does not take place.
Mucoid, mucus-like, referring specifically to a bacterial phase producing slimy growth.
Mycelioid, colonies having the radiately filamentous appearance of mold colonies.
Napiform, liquefaction in form of a turnip.
Ontogenetic, pertaining to the life history of an individual.
Opalescent, milky white with tints of color as in an opal.
Opaque, not allowing light to pass thru.

Optimum temperature, temperature at which most growth occurs.

Papillate, growth beset with small nipple-like processes.

Parasitic, deriving its nourishment from some living animal or plant upon which it lives and which acts as host; not necessarily pathogenic.

Pathogenic, not only parasitic (q. v.) but also causing disease to the host.

Pellicle, bacterial growth forming either a continuous or an interrupted sheet over the culture fluid.

Peptonization, rendering curdled milk soluble by the action of peptonizing enzymes.

Peritrichiate, applied to the arrangement of flagella, indicates that they are distributed over the entire surface of an organism.

Peritrichic, having flagella in peritrichiate arrangement.

Per os, thru the mouth.

Persistent, lasting many weeks or months.

Phase variation, separation of a species into strains, having somewhat different characters.

Photogenic, glowing in the dark, phosphorescent.

Polar, at the end or pole of the bacterial cell.

Precipitin, an antibody having the power of precipitating soluble proteins.

Pulvinate, cushion-shaped.

Punctiform, very small, but visible to naked eye; under 1 mm. in diameter.

Raised, growth thick, with abrupt or terraced edges.

Reduction, removing oxygen or its equivalent from a chemical compound; or addition of hydrogen or its equivalent. Refers to the conversion of nitrate to nitrite, ammonia, or free nitrogen; also to the decolorization of litmus.

Rennet curd, coagulation of milk due to rennet or rennet-like enzymes, distinguished from acid curd by the absence of acid.

Rhizoid, growth of an irregular branched or root-like character, as *B. mycoides.*

Ring, growth at the upper margin of a liquid culture, adhering to the glass.

Rugose, wrinkled.

Saccate, liquefaction in form of an elongated sac, tubular, cylindrical.

Saprophytic, living on dead growth in the absence of organic matter, i. e., neither autotropic (q. v.) nor parasitic.

Sensitize, to render sensitive, usually to a foreign protein.

Sepsis, a state of infection.

Sheath, an envelope similar to a capsule (q. v.), but surrounding a filamentous organism.

Spindled, larger at the middle than at the ends. Applied to sporangia, refers to the forms frequently called clostridia.

Sporangium (*pl.*-**ia**), cells containing endospores.

Spreading, growth extending much beyond the line of inoculation, i. e., several millimeters or more.

Stratiform, liquefying to the walls of the tube at the top and then proceeding downwards horizontally.

Strict aerobe, see *aerobic.*

Strict anaerobe, see *anaerobic.*

Subcutaneous, under the skin.

Subterminal, situated toward the end of the cell but not at the extreme end, that is between the positions denoted excentric (q. v.) and terminal.

Synergism, coöperative action of two organisms, resulting in an end-product which neither could produce alone.

Thermophilic, growing best at high temperatures, i. e. 50°C or over.

Toxic, poisonous.

Transient, lasting a few days.

Translucent, allowing light to pass thru without allowing complete visibility of objects seen thru the substance in question.

Trituration, thoro grinding in a mortar.

Truncate, ends abrupt, square.

Turbid, cloudy with flocculent particles; i. e., cloudy plus flocculence.

Ulcer, an open sore.

Undulate, wavy.

Villous, having short, thick, hair-like processes on the surface, intermediate in meaning between papillate and filamentous.

Virulence, degree of pathogenicity (referring to infectiousness).

Virus, a self-propogating cause of disease, often referring to one too small to be seen with microscope.

Viscid, growth follows the needle when touched and withdrawn; sediment on shaking rises as a coherent swirl.

LEAFLET II

PREPARATION OF MEDIA

9th EDITION
Pure Culture Study of Bacteria, Vol. 12, No. 2
APRIL, 1944

Including a section prepared by
Committeeman on Anaerobic Methods

LEAFLET II

PREPARATION OF MEDIA

STERILIZATION

General directions for preparation of media are hardly called for here as they can be found in all bacteriological laboratory guides. In the matter of sterilization, however, a few specific instructions seem advisable.

Ordinary bacteriological media are sterilized for 20 to 30 minutes in an autoclave under steam pressure at 121°C (15 pounds pressure after driving out all air). In determining this temperature dependence should not be laid upon a pressure gauge; the autoclave should be equipped with a thermometer. In general, the smaller the container, and the smaller the number of flasks or tubes sterilized at one time, the shorter the sterilizing time can be. In the case of small batches of media, 15 minutes at 15 pounds are ordinarily sufficient, a fact which is worth taking into account when the media contain substances likely to be decomposed by heat.

Oils are difficult to sterilize, and when they are added to media it is well to sterilize them separately by dry heat (165-75° for 1 hour) or by autoclaving *in small quantities* at 121°C.

Fractional sterilization in flowing steam at 100° for 30–60 minutes on three successive days was formerly recommended to avoid this decomposition in the case of carbohydrates. Recent investigation, however, tends to show that this procedure can be more harmful than the higher temperature for 15 minutes; fractional sterilization, therefore, is used much less than formerly. Instead it is recommended that those sugars especially susceptible to the effects of heat (e. g., xylose, arabinose, fructose, maltose, and under some conditions sucrose and lactose) be dissolved separately and sterilized by filtration before adding to the rest of the medium after it has been autoclaved. The Seitz filter or sintered glass filters prove suitable for this purpose. Where facilities for such filtration are lacking, these sugars can ordinarily be autoclaved successfully if sterilized separately from the rest of the medium and in concentrated solution, employing as brief heating as possible—e. g., 10 minutes at 10 pounds pressure (115°C) if serological tubes are used.

MEDIA USED IN PURE CULTURE STUDY

It is a matter of some difficulty to decide just what media should be included here. It would obviously be beyond the scope of this

Manual to include all the media employed by bacteriologists. In selecting the ones to include two principles have been kept in mind: first to include only those known to be in fairly common use among American bacteriologists; second, realizing that this is a Manual for *Pure Culture Study* of Bacteria, not to list media that are used purely for counting bacteria or for the diagnosis of disease. The media given here are employed either for maintaining pure cultures or for the identification of species.

For the purposes of this Manual these media may be classified as follows: *A.* Cultivation and storage media; *B.* General differential media—i.e. media employed in tests for determining the identity of saprophytic aerobes, in general; *C.* Media for special groups of aerobes—i.e. media employed in the identification of bacteria of certain narrow groups, such as the colon-typhoid group; *D.* Media for anaerobic bacteria.

The media included in this leaflet under the heading "*C*" have been arranged into three groups the first of which is denoted "Basal Media". The basal media may be considered as formulae to which substances under investigation (e. g., sugars) may be added. The selection of any of these basal media depends upon the group of bacteria to be studied.

Special reference is made here to Levine and Schoenlein's Compilation of Culture Media, 1930. In the case of the formulae taken from this source, the number therein assigned to the medium is given at the head of each formula under the designation "L&S No. . . ."

Attention is called to the fact that many of these media are now on the market in dehydrated form. Use of such dehydrated media is entirely permissible, and often convenient.

A. Cultivation and Storage Media

Beef-extract broth ordinarily has the following composition:

Beef-extract	3 g.
Peptone	5 g.
Distilled water	1000 ml.

Concerning the peptone called for in the above formula, no definite specifications can yet be given. Various commercial products are available, no one of which is superior for all bacteriological purposes. In the case of reports on standard methods (e.g., those of the American Public Health Assoc.) emphasis is laid on one brand of peptone

for the sake of uniformity; for purposes of pure culture study, however, any brand known to give best results for the purpose at hand may be employed.

Beef-extract agar may be of the same composition plus the addition of 12 grams of oven-dried agar or 15 grams of commercial agar. The agar is to be dissolved by heat (e.g. autoclaving) and the sediment removed either by decantation or by filtration through cotton.

Beef-extract gelatin may be of the same composition as the broth but with the addition of 100 g. of "Bacto-gelatin" (or some other gelatin of the same jellying strength; i.e., 175-200 by Bloom test). Carefully adjust reaction (see below) after dissolving gelatin and heat for 5 or 10 min. at about 100° C. Filter through cotton.

Meat infusion broth. This is usually prepared as follows: Pour 1 litre of water over 400–600 g. of lean beef or veal, ground through a meat chopper. Allow to stand in a refrigerator overnight and then skim off the scum of fat with a piece of absorbent cotton. Squeeze out the infusion through a strong muslin cloth and make the amount up to 1000 ml. Dissolve 5 g. of peptone in this by adding the infusion (without heating) little by little to the peptone in a mortar and rubbing up with the pestle until the solution is complete. (When making this bouillon as a basis of blood agar or for serological work, one should also dissolve in it 0.5 g. sodium chloride.) Adjust reaction (see below). Heat for about 20 minutes at about 100°C without stirring; filter through wet filter paper and make up to 1000 ml.

Meat infusion agar. In 1000 ml. meat infusion broth prepared as above, dissolve 12 g. of oven dried agar (or 15 g. commercial agar) by heating at about 100°C; filter off any sediment formed.

Yeast-extract broth and agar. These may be made the same as beef-extract broth and agar except for replacing the beef-extract with 2.5 g. yeast-extract per litre. The latter should be used in powdered form, as for example the product of the Difco Laboratories.

Semi-solid agar. With some organisms, especially microaerophiles, more successful cultivation can be obtained by means of semi-solid media, containing only 0.2 to 0.5% agar. For such purposes any of the above agar formulae may be followed, merely decreasing the quantity of agar. The exact quantity of agar recommended varies. Thus Hitchens' semi-solid medium (see p. 11[44]-11) calls for 0.2%, while Tittsler and Sandholzer (1936) employ a 0.5% agar for the macroscopic determination of motility: the latter is almost solid in consistency.

Adjusting reaction. The reaction of all these media is to be adjusted to a hydrogen-ion concentration near neutrality (i.e. pH 7.0). The beef-extract broth and agar normally require no adjustment to bring them to this reaction; the others need the addition of alkali ordinarily. In all cases the reaction should be tested, even though no adjustment is thought to be necessary. For detailed instructions in testing or adjusting the reaction one may consult Leaflet IX of this Manual, entitled The Determination of pH and Titrable Acidity or may follow the directions given by the American Public Health Association (1936, p. 199). For ordinary purposes, however, good results will be obtained by adjusting the media to the neutral point of brom thymol blue;[1] the medium is brought to such a reaction as to turn this indicator a distinct grass-green (neither yellow green nor blue green). This color corresponds closely to the desired reaction. Another equally satisfactory method to bring the medium to this reaction is to add sufficient alkali to cause the first faint trace of permanent pink to appear with phenol red.[1] Reaction should always be checked after final sterilization of each batch.

Natural storage media. Recent years have shown quite a tendency to employ natural media, particularly skim milk or soil, for the storage of stock cultures. These materials are frequently used in their natural state, without addition; but more often a small quantity of calcium carbonate is added to neutralize acids formed. This addition is absolutely necessary in the case of limestone-free soils or in the case of milk when the organisms to be stored produce acid from lactose.

B. General Differential Media

Plain gelatin for use in the determination of gelatin liquefaction. This is made up like beef-extract gelatin but without the beef-extract and peptone; it consists of 10% "Bacto-gelatin" (or some other brand of the same jellying strength) dissolved in distilled water and the reaction adjusted to pH 7.0.

Sugar broths. Just before sterilization 0.5–1% of the required carbohydrate is ordinarily added to beef-extract broth; the same proportions are also usually to be employed in studying the fermentation of any related carbon compound (e.g. alcohol or gluco-

[1]Use 0.04% brom thymol blue or 0.02% phenol red. Alcoholic solutions may be employed without neutralizing, or aqueous solutions of the sodium salts prepared as directed by Clark (1928, p. 91–95) or as explained in Leaflet IX of this Manual (p. IX_{41}-11).

side). The final reaction should be adjusted to pH 7.0. For precautions in sterilization, see above, p. II41-3.

It is often desirable to put some indicator into such media. In selecting the proper indicator read the section below on Indicator Media.

Sugar agar. As with sugar broth, beef-extract agar media of the formula given on p. 5 may be made up with 1% of the required carbohydrate or related carbon-compound. The latter may be mixed with the other ingredients only if it is known not to be appreciably changed by the heat employed; otherwise it should be dissolved and sterilized separately as above suggested. The reaction should be adjusted to pH 7.0. An indicator may be added if desired.

Indicator media. Carbohydrate media with some indicator to show acid production are frequently of value. Litmus and Andrade's indicator (acid fuchsin decolorized with alkali) are much used, but they do not give accurate results in terms of hydrogen-ion concentration; so, except for certain special purposes[2], it is recommended that sulphonphthalein indicators be employed. The indicators of most value are: phenol red, brom thymol blue, brom cresol purple, brom cresol green, and occasionally brom-chlor phenol blue. Their use is governed by the following considerations:[3]

Phenol red indicates changes to the alkaline side of neutrality, as its range is pH = 6.8–8.4. For use in indicator media it is best kept in a 1.6% alcoholic solution and 1 ml. of the solution added to 1 litre of medium.

Brom thymol blue has a sensitive range extending slightly in either direction from neutrality. It is useful in media carefully adjusted to pH 7.0, but indicates such small changes in reaction as to be often impractical. It is best added to media at the rate of 1 ml. of a 1.6% alcoholic solution to the litre.

Brom cresol purple indicates slightly greater changes to the acid side of neutrality, as its range is pH = 5.2–6.8. For indicator media 1 ml. of a 1.6% alcoholic solution should be added to the litre. It seems to be the most generally useful indicator for indicator media of any at present available. It has, however, the defect of dichromatism. If this is troublesome, it may be replaced by brom phenol red, which covers the same pH-range.

Combinations of brom cresol purple and cresol red are often satisfactory when looking for changes in either direction from neutrality. When this combination is employed, the media should be

[2]See next page; also Leaflet V, p. V42-20

[3]See also Leaflet IX.

carefully adjusted to pH 7.0 with brom thymol blue before adding any indicator; then 1 ml. of a saturated aqueous solution of each indicator should be added. This mixture of indicators changes very slowly from purple to yellow through a long range (from about pH = 8.0 to about pH = 5.2) extending for a considerable distance on each side of neutrality. By comparing with a blank tube of the neutral medium it is easy to detect an increase either in acidity or in alkalinity.

Brom cresol green (introduced by Cohen, 1922) indicates moderately great changes to the acid side of neutrality as its range is pH = 3.8-5.4. It is best kept for this purpose in a 2% alcoholic solution, adding 2.0 ml. to each litre of medium. Used in agar media it shows appreciable change from green to yellow if the reaction is as high as pH = 5.2; and from that point to the acid end of its range it is very satisfactory.

Brom phenol blue is now suggested by Cohen (1927) to replace brom-chlor-phenol blue which he described earlier. As its range is from pH = 3.0 to pH = 4.6 it is of value in indicator media only with organisms showing a very high final hydrogen-ion concentration. For this reason it is very seldom called for; but it is valuable in distinguishing the most vigorous acid formers.

In spite of all the arguments in favor of the sulphonphthaleins as H-ion indicators, litmus still remains popular among bacteriologists, and no perfect substitute for it has been obtained. Its advantages are that it is a long-range (even if not highly accurate) indicator, showing changes on both sides of the neutral point, and at the same time indicates changes in oxidation-reduction potential. This makes it useful for diagnostic purposes when employed in certain media, notably in milk; and no combination of indicators showing all the characteristics of litmus has yet been proposed. Unfortunately, at the time when this (9th) edition of this leaflet goes to press, the source of the lichens from which litmus is manufactured has been cut off from the United States, and this indicator is becoming harder and harder to obtain.

Nitrate broth. For routine work 0.1% KNO_3 is added to the regular formula for beef-extract broth and reaction adjusted as usual. Similarly routine *nitrate agar* should contain 0.1% KNO_3 added to the ordinary formula for beef-extract agar, with the reaction properly adjusted. Modification of these formulae is often necessary as explained on p. V42-10 Leaflet V of this Manual. A synthetic nitrate medium often found useful is given below (p. II44-14).

Media for H_2S production. In previous editions, four media have been listed containing lead or iron salts, designed to show blackening when hydrogen sulfide is produced. As the present procedure given in Leaflet V calls for lead acetate test-strips in the mouths of the tubes, these media are no longer recommended for routine use. Those who wish to use such media are referred to the papers of Bailey and Lacy (1927) and of Wilson (1923), who describe lead and iron salt media, respectively; or they may consult the manual of the Difco Laboratories, who manufacture dehydrated media for the purpose in question.

Churchman's gentian violet agar for selective bacteriostasis. To ordinary beef-extract-peptone agar add a definitely determined amount of crystal violet of about 85% dye content. If the medium is to be used to inhibit Gram-positive organisms and permit the growth of Gram-negatives the dye concentration should be about 1:100,000. If it is to be used for differentiation between the Gram-positives its concentration should be between 1:400,000 and 1:800,000; if for differentiation between Gram-negatives it should be between 1:1,000 and 1:40,000. In either of the two latter cases the exact concentration depends upon which particular bacteria it is desired to inhibit and which to permit to grow.

C. Media for Special Groups of Aerobes

1. BASAL MEDIA

Douglas trypsin broth (Hartley) (L&S No. 1123). Mix 150 g. of lean minced horse meat with 250 ml. tap water and heat at 80°C in a steamer. Add 250 ml. of an 0.8% Na_2CO_3 (anhydrous) and cool to 45°C. Add 5 ml. of chloroform and 5 ml. of pancreatic extract prepared as directed by Cole and Onslow (1916) and Douglas (1922).

Preparation of pancreatic extract: To 1000 g. minced fresh pig pancreas (free from fat) add 3000 ml. distilled water and 1000 ml. 95% ethyl alcohol. Place in a large clean bottle; shake repeatedly; and allow to stand 3 days at room temperature. Strain through gauze and filter through paper. (Filtration is slow.) Add 1 ml. conc. HCl. to each 1000 ml. of filtrate. This causes a cloudy precipitate which settles in a few days and can be filtered off. The liquid keeps indefinitely if placed in a stoppered bottle; no additional antiseptic is needed.

Estimation of activity: Centrifuge fresh milk and discard the cream; add 1% $CaCl_2$. Make a series of dilutions (1:100, 1:200, 1:500, 1:1000, 1:2000, 1:4000, etc.) of the pancreatic extract, and place in tubes, 1 ml. to the tube. To each tube add 1 ml. of the milk. Place in a water bath at 50°C for 30 min. The highest dilution of trypsin which causes clotting is a measure of its potency. Alcoholic pancreatic extract usually causes clotting at 1:1000; Bacto-trypsin at 1:5000.

Incubate at 37°C for 6 hours, shaking frequently. Add 40 ml. normal HCl and heat in the steamer for 30 minutes. Cool and filter. Adjust to pH 8.0. Distribute as desired.

Pass steam through the autoclave for one hour then raise the pressure slowly to 10 pounds and turn off the steam. For sterilization of larger quantities (one litre in a flask) maintain the pressure at 10 pounds for 30 minutes.

Use: Preparation of diphtheria toxin, for growth of numerous pathogens, and as medium for blood culture.

Kracke and Teasley medium. Dissolve 500 g. finely ground fat-free heart muscle in 1000 ml. water. Place in ice-box overnight. Filter through four layers of gauze, heat to boiling, and filter through fine wire mesh or copper gauze.

Mix separately 500 g. finely ground brain in 1000 ml. water. Place in ice-box over night. Filter and heat slowly to boiling; keep stirring. Do not filter after heating.

Prepare medium as follows:

75 ml. heart muscle extract	1 g. glucose
25 ml. brain suspension	1 g. peptone
0.1 g. sodium citrate ($Na_3C_6H_5O_7+2H_2O$)	0.5 g. Na_2HPO_4 (anhydrous)

Heat until ingredients are in solution, adjust to pH 7.4, autoclave at 15 pounds for 15 minutes.

Use: Blood culture of pathogens.

Ascitic fluid agar. Various formulae have been proposed. A simple one is as follows:

Melt 100 cc. of sterile Douglas' agar, pH 7.4–7.8, in a flask. Cool to about 48° to 50°C. With sterile pipette add 20 ml. of sterile, bile-free, ascitic fluid. Pour into tubes or plates and allow to harden.

Use: Cultivation of pathogenic cocci.

Loeffler's blood serum. A common formula for this calls for glucose beef-extract broth as its basis. This is prepared as follows:

Beef extract	3 g.	Peptone (Difco or Witte)	10 g.
Glucose	10 g.	NaCl	5 g.
Distilled water			1000 ml.

Mix the ingredients and dissolve by warming over a flame. Do not adjust the reaction. Filter through paper. When the broth is cool add one volume to three volumes of clear serum of horse, beef, or pig. Tube, 3 to 4 ml. per tube, and place tubes in a slanting position in a pan or rack. Take care to prevent the occurrence of bubbles and frothing. Cover tubes with newspaper. Sterilize in autoclave at 15 pounds for 15 minutes without letting the air out, and repeat on two successive days. Or if it is pre-

ferred to complete the sterilization in one day, heat for 15 minutes at 15 pounds without letting the air escape; then let the air escape slowly while maintaining pressure, after which the air vent should be closed and sterilization continued at 15 pounds for 15 minutes longer. After completion of the sterilization the pressure should be allowed to fall very slowly.

Use: Cultivation of diphtheria organism.

Huntoon's Hormone Heart Infusion Broth (*L&S No. 837*). Mix 10 g. Bacto peptone, 10 g. gelatin, 5 g. NaCl, one whole egg and 500 g. of finely chopped beef heart in a litre of water. Place in an enamel-ware vessel, *e.g.* a large coffee pot. Heat over a free flame with constant stirring until the red color of the meat infusion changes to brown at a temperature of about 68°C. Do not go beyond this temperature. Adjust to slightly alkaline to litmus and then add 1.0 ml. additional *N*/1 NaOH per litre of medium. Cover the vessel and place in an Arnold sterilizer or in a water bath at 100° for one hour. Remove the vessel from the sterilizer and separate with a glass rod the firm clot which has formed from the side of the vessel. Return to the Arnold sterilizer at 100° for 1½ hours. Remove the vessel and allow to stand at room temperature for about 10 minutes in a slightly inclined position. Pipette off the fluid portion or decant. If it is poured through a fine wire sieve, many of the fine pieces of meat clot may be caught. (Avoid filtering through cheese cloth, cotton or other absorbent materials.) Allow it to stand in tall cylinders for 15 to 20 minutes until the fat present has risen to the surface and been removed. The medium may be further cleared by filtering through glass wool, asbestos wool, sedimentation or centrifugation. Add 0.15% dextrose and enough laked blood to give a slight pink tint. Tube in 10 ml. lots. Sterilize by the intermittent method.

Use: To cultivate highly pathogenic organisms.

Hitchens' Semi-solid Glucose Agar (*Mulsow*) (*L&S No. 879*). Add 500 ml. of water to 1 pound of ground lean beef, and allow to stand at 37°C. for 48 hours. Express the juice and add 20 g. peptone, 2 g. KNO_3 and an equal amount (500 ml.) of a 0.2% agar solution heated and cooled to 60°C. Adjust the reaction to + 0.9 to phenolphthalein. Heat in the autoclave at 15 pounds pressure for 25 minutes. Filter and readjust the reaction if necessary. Add 2 g. glucose. Final method of sterilization not specified.

Use: Cultivation of gonococcus and microaerophilic bacteria in general.

Egg Medium with Glycerol. Break several eggs into a graduated cylinder. Add ⅓ as much meat infusion or Douglas' broth as the amount of eggs. Add 1% glycerin. Stir to mix, taking care not to cause the formation of air bubbles or frothing. Filter through gauze. Tube and slant in a pan or rack. Sterilize in the autoclave at 15 pounds for 15 minutes.

Use: Cultivation of tubercle organism.

2. DIFFERENTIAL MEDIA

Endo medium. Dissolve 5 g. beef extract and 10 g. peptone in 1000 ml. water. Add 30 g. agar and cook in autoclave 45 minutes at 15 lbs. pressure. Filter; then add 10 g. lactose and sterilize 15 minutes at 10 lbs. pressure in small containers 100 ml. in each. Just before use prepare a 3% solution of basic fuchsin (85–90% actual dye

content) in 95% ethyl alcohol. Add 1 ml. of this fuchsin solution to 100 ml. of the agar (melted) also 0.125 g. anhydrous sodium sulfite dissolved in about 5 ml. distilled water and pour plates immediately. The medium should be light pink while hot and almost colorless after cooling; as varying results may be obtained with different batches of fuchsin, it is sometimes necessary to use a weaker stock solution of that dye (e. g., 2% or occasionally only 1%).

Brilliant-green-bile medium. Dissolve 20 g. dried oxgall and 10 g. peptone in 1000 ml. boiling water; cook in a double boiler or steam for an hour. Add 10 g. lactose, and filter through cotton or cotton flannel. Adjust reaction to between pH 7.1 and 7.3. Add 0.013 g. of brilliant green (85–90% dye content). This concentration of bile and dye is adjusted to permit the growth of bacteria of the colon-aerogenes group, but to restrain or prevent the growth of Gram-positive organisms, which often confuse diagnostic routine. When used in water analysis, and more than 1 ml. of water is added to each tube, the medium should be made of sufficiently greater concentration so that the final dilution will be the same as that above indicated.

Levine's eosin-methylene-blue agar. Dissolve by boiling:

Distilled water	1000 ml.	K_2HPO_4	2 g.
Peptone	10 g.	Agar	15 g.

Before sterilizing add to 100 ml. of the above: 2 ml. sterile 2.0% aqueous solution eosin Y (dye content about 85%), and 2 ml. sterile 0.325% aqueous methylene blue (dye content about 85%). Just before use add aseptically 5 ml. sterile 20% lactose solution. Reaction not adjusted. Do not filter.

Buffered peptone solution for methyl red and Voges-Proskauer tests. Dissolve 7 g. peptone (Witte or Difco Proteose Peptone), 5 g. glucose and 5 g. K_2HPO_4 in 1000 ml. distilled water. Adjust reaction to pH 6.9–7.0, and sterilize in the autoclave.

Blood broth. Add 5% of rabbit, sheep, or horse blood, drawn aseptically and defibrinated, to beef extract broth or meat infusion broth.

Blood agar. Prepare beef extract or meat infusion agar containing 2% (instead of 1.2%) agar. Melt 100 ml. of this, cool to 45°C, and add 5 ml. of rabbit, sheep, or horse blood, drawn aseptically and defibrinated. The medium should be poured into plates or slanted in tubes very soon after adding the blood.

Bismuth-sulphite agar (Wilson and Blair, 1926; formula from Diagnostic Procedures and Reagents, A. P. H. A., 1941, p. 25). To 1 litre nutrient agar (2% agar, 0.5% beef

extract, and 1% peptone) add 45 ml. of 1% aqueous ferric citrate containing 11% of 1% aqueous brilliant green, also 200 ml. of bismuth sulfite mixture prepared as follows: dissolve 6 g. bismuth ammonium citrate scales in 50. ml. boiling water, and 20 g. anhydrous Na_2SO_3 in 100 ml. boiling water, mix, bring to a boil, and dissolve 10 g. anhydrous Na_2HPO_4 in the mixture while boiling, cool and add 10 g. glucose dissolved in 50 ml. boiling water, restore lost water. After mixing these two solutions with the melted agar pour immediately into petri dishes; after 1–2 hr. at room temperature these plates may be stored in a refrigerator, but must be used within 4 days.

Use: Enrichment of typhoid and paratyphoid groups.

Tellurite agar. (Anderson, et al. 1931). Add 1½ to 2 lbs. minced meat to 1000 ml. tap water at 48° C; after an hour squeeze out juice through cloth, leave in refrigerator overnight and filter through filter paper. To 1000 ml. filtrate add 20 g. peptone and 5 g. NaCl and dissolve at 45° C. Adjust reaction to pH 7.6. Filter first through a Seitz K clarifying film; then sterilize by filtration through a sterile Chamberland candle, collecting in sterile flasks and tubes. Incubate a few tubes for a check on sterility but store the rest in a refrigerator. For use, mix with equal parts of 5% sterile agar in water. Add 7–10% freshly drawn defibrinated rabbits' blood and 0.01% potassium tellurite. Heat at 75° C for 10–15 minutes before pouring into plates.

Use: Differentiation of diphtheria organism.

Desoxycholate agar. (Liefson, 1935).

Water			1000 ml.
Peptone	10 g.	Ferric ammonium citrate	2 g.
Agar	12–17 g.	K_2HPO	2 g.
NaCl	5 g.	Sodium desoxycholate	1 g.
Lactose	10 g.	Neutral red (1% aqu. sol.)	3 ml.

Dissolve the peptone in the water, adjust to pH 7.3–7.5, boil briefly and filter through paper. Add the agar and dissolve by autoclaving; add 6 ml. of *N* NaOH, then the other ingredients in the order named, omitting the neutral red until after a final adjustment of the reaction to 7.3 or 7.5 as desired. Sterilize by heating in flowing steam only long enough (i.e. about 15 minutes) to kill vegetative cells.

Use: Isolation of colon organisms from milk.

Desoxycholate-citrate agar. (Liefson, 1935). Mix 333 g. fresh, lean, ground pork with 1000 ml. distilled water and allow to infuse for about an hour; add 3.3 ml. *N* HCl and boil for about one minute; filter through paper and add 3.3 ml. *N* NaOH; boil for one minute and filter through paper; bring volume up to 1000 ml. by adding distilled water. Add 10 g. peptone and adjust reaction to about pH 7.5. Boil 2–3 minutes and filter through paper; then add 20 g. agar and 5 ml. *N* NaOH; after at least 15 minutes standing, melt agar by boiling or autoclaving. Add as rapidly as possible in the following order: 10 g. lactose, 25 g. sodium citrate ($Na_3C_6H_5O_7+5½H_2O$), 3.5 mg. $PbCl_2$ (optional). Just before using, and while melted and held at a temperature of 80–100° C, add 0.2% ferric ammonium citrate (green scales); adjust reaction to pH 7.4. and add to each 100 ml. 0.2 ml. of 1% aqueous neutral red. (It is important that the temperature of the medium at the time should be high enough to kill vegetative cells.) Pour into plates without further sterilization.

Use: Isolation of typhoid organism from milk.

3. SYNTHETIC MEDIA

Ashby's mannitol solution. In one litre of distilled water dissolve the following:

Mannitol	20.0 g.	NaCl	0.2 g.
K_2HPO_4	0.2 g.	$CaSO_4+2H_2O$	0.1 g.
$MgSO_4+7H_2O$	0.2 g.	$CaCO_3$	5.0 g.

Method of sterilization not specified by author; autoclaving presumably satisfactory.

Use: Cultivation of *Azotobacter.*

Synthetic carbohydrate media. Peptone-free media are often valuable in measuring increases in hydrogen-ion concentration when only small quantities of acid are produced. A formula slightly modified from one proposed by Ayers, Rupp and Johnson (1919) is as follows:

$NH_4H_2PO_4$	1.0 g.	Adjust to pH 7 by the addition of NaOH. About 6 ml. normal NaOH required.
KCl	0.2 g.	
$MgSO_4+7H_2O$	0.2 g.	
Water	1000 ml.	
Sugar (or other carbon source)	10 g.	

This may be employed as a liquid medium without or with the addition of indicator; or as a solid medium with the addition of 15 g. of air-dry agar. Used with agar for the detection of acidity, it is necessary to have an indicator present.

Synthetic nitrate medium. A modification of the above is valuable in detecting nitrate reduction in the case of some organisms that do not produce nitrite from nitrate in a peptone medium.

K_2HPO_4	0.5 g.	To prevent precipitation of calcium phosphate, one or the other of the first two salts listed should be dissolved separately in a portion of the water and added after the other ingredients have been brought into solution. No adjustment of reaction required.
$CaCl_2$ (anhyd.)	0.5 g.	
$MgSO_4+7H_2O$	0.2 g.	
Glucose	10 g.	
KNO_3	1 g.	
Distilled water	1000 ml.	

D. MEDIA FOR ANAEROBIC BACTERIA[4]

Before listing the various media which are to be used for anaerobic bacteria, it is necessary to introduce briefly the related topic—oxidation reduction (O/R) potential.

The O/R potential required for obligate anaerobes is in general low (Hewitt (1937), Knight (1931), and Reed and Orr (1943).) The usual fluid medium is a complex of active oxidation-reduction systems, but if the medium is prepared from peptone or more simple constituents, usually it is necessary to include special substances to bring the potential to the desired low level. The addition of a small amount (0.1%) of agar will aid in the prevention of diffusion of atmospheric oxygen into the medium, but this

[4]This section has been prepared for the Committee by L. S. McClung.

is not sufficient aid for many species. If other actively reducing substances are necessary, the following are the most suitable: glucose, sodium thioglycollate (and thioglycollic acid), sodium formaldehyde sulfoxylate, ascorbic acid, sodium formate, glutathione, and cysteine. Glucose seems to be generally satisfactory, but some of the other compounds are toxic for certain types. Methylene blue (1–500,000) may be added to culture media to serve as an O R indicator. Obligate anaerobes will grow *only* in the portion in which the dye remains decolorized following cooling after sterilization.

The spore-forming anaerobes frequently have been divided into proteolytic and saccharolytic groups. An organism of the former group possesses the ability to decompose complex proteins, usually with the production of offensive odors, sometimes attacking a small variety of the simpler carbohydrates. The saccharolytic group, on the other hand, usually show little action on complex proteins (except such compounds as gelatin), but ferment a wide variety of the carbohydrates, usually with copious production of gas.

Reference in this Leaflet is also made to the "pathogenic group" and the "butyric-butyl group". The former term is used to designate such organisms as *Clostridium tetani*, *C. septicum*, *C. histolyticum*, *C. chauvoei*, *C. perfringens*, (*C. welchii*), *C. sporogenes*, and *C. parabotulinum*, etc., which grow best in the richer animal tissue infusions and require a high degree of anaerobiosis. Representatives of the butyric-butyl group include *C. butyricum*, *C. beijerinckii*, *C. butylicum*, *C. pasteurianum*, *C. acetobutylicum*, *C. felsineum*, *C. roseum*, and *C. thermosaccharolyticum*; they are less exacting with regard to oxygen exclusion and grow best when supplied a fermentable carbohydrate. Due to the diversity of physiological types within the anaerobic group it will be necessary frequently to recommend two or more media for the same purpose.

All liquid media (except the thioglycollate medium and the semi-solid corn liver medium) should be boiled 10 minutes, or heated in flowing steam for a similar period, immediately prior to inoculation unless the medium is used on the same day it is initially sterilized. The use of vaseline, mineral oil, or other seals at the surface of liquid media is not recommended. If a liquid medium is used which will not remain reduced during the desired incubation period, incubate the tubes in an anaerobic jar (see Leaflet III, *4th Ed.*).

1. ENRICHMENT AND GENERAL CULTIVATION MEDIA

Dehydrated Thioglycollate Medium[5]. This medium (Brewer, 1940a, b) is obtained in dehydrated form from the manufacturers. After dissolving, it is essentially a liquid (the percentage of agar being too small to affect the fluidity) in which sodium thioglycollate acts as a reducing agent. It also contains meat infusion, peptone, NaCl and a phosphate, with or without glucose and methylene blue; for most purposes the presence of these last two ingredients is recommended. The medium compares favorably with other infusion media in ability to initiate growth from small inocula (McClung, 1940, 1943).

The appropriate amount (indicated on bottle) of the dry powder is dissolved in distilled water by brief heating, tubed or dispensed in

[5]*Dehydrated thioglycollate medium.* Baltimore Biological Laboratory, Baltimore, Maryland, or Difco Laboratories, Detroit, Michigan. If the commercially prepared medium is not available, a satisfactory substitute can be prepared by adding 0.1% agar and 0.1% sodium thioglycollate to a meat infusion base medium.

deep columns in flasks or bottles, and sterilized 20 minutes at 15 lbs. pressure. Upon cooling, if methylene blue is present, a greenish blue color should develop at the surface and sometimes to some distance below the surface if the medium is disturbed; upon standing a short time, however, the usual amber color indicative of anaerobiosis will return. The medium may be stored (*at room temperature, not in a refrigerator*) for several days, or even a few weeks, and used without the heating, required by most media, to expel absorbed oxygen.

Use: Recommended as the medium of choice in the enrichment of the pathogenic anaerobes. Particularly useful in hospital laboratories where small amounts may be made as needed from the dry powder. Not recommended for isolation of the butyric-butyl group. Since it is claimed that the thioglycollate not only maintains a low O/R potential, but also combines with and inactivates most of the mercurials, (Daily and Blubaugh, 1941; Blubaugh and Reed, 1943; Nungester *et al.*, 1943), this medium is suggested for use in the routine sterility testing of biological materials including vaccines, serums, catgut, etc. (Marshal *et al.*, 1940; Federal Register, 1942).

Beef Heart (*or beef tissue*) *Infusion Medium.* Several different formulae are available for this medium; although there seems to be little reason to choose any particular one, in preference to another, the following is satisfactory: Allow 500 g. of beef heart (or lean beef meat) to stand overnight in refrigerator in 1,000 ml. of tap water. Trim fat from the meat, and mince or grind before adding to the water. Remove from icebox and boil over free flame for 15 minutes or steam in Arnold sterilizer for 30 minutes. Separate tissue from liquid by passing through two layers of cheese cloth in a fluted glass funnel, and save both portions. Add 10 g. peptone and 5 g. NaCl to the liquid after restoring to volume. If necessary, heat briefly to dissolve peptone. Adjust to pH 7.6 with 1 *N* NaOH and boil for 15–20 minutes or heat in Arnold sterilizer for 30 minutes. Filter through paper. If needed immediately, tube broth over a 2 cm. column of tissue, and sterilize 45 minutes at 15 pounds pressure. If not needed immediately, sterilize broth in screw-capped bottles, and rapidly dry tissue in incubator with forced circulation. These may be used at any later time. Check the sterility of the medium before use by incubation for at least 24 hours at 37° C.

Use: For enrichment or general cultivation of pathogenic anaerobes; not suitable for the butyric-butyl group of the thermophilic anaerobes. Has some diagnostic value as certain species produce a reddening of the tissue. (Strongly proteolytic organisms cause a disintegration of the meat tissue with the release of offensive odors.) Suitable for stock cultures of most of the pathogenic types, as in most instances (exception *C. perfringens*) spore production may be detected after 48 hours. Certain proteolytic species deposit crystals of tyrosine in this medium upon extended incubation.

Beef liver infusion medium. Remove fat from 500 g. of fresh beef liver, grind, and heat, with occasional stirring, in 1,000 ml. of tap water for one hour in the Arnold sterilizer. Cool and strain through cheese cloth. Restore filtrate to original volume and add 1% peptone and 0.1% K_2HPO_4. Dry tissue (at 55° C. if available) as rapidly as possible. Tube broth over several chunks of tissue. Use the broth (before addition of peptone and phosphate) in the original strength, or diluted five times. Sterilize 30 minutes at 15 lbs. pressure. Avoid longer heating of medium as this diminishes its value with respect to initiation of growth from small inocula.

Use: Recommended especially for enrichment, from spore stocks or other sources, of the butyric-butyl group and *C. perfringens.* May replace beef heart medium for pathogenic types. Useful for enrichment medium in detection of thermophilic contamination of sugar, starch, canned foods, etc. (Sometimes difficulty is encountered with this medium and the following one due to a Gram-positive rod which develops as a contaminant during the drying of the liver tissue.)

Corn Liver Medium. Add 50 g. of ordinary (white or yellow) corn meal and 10 g. of dried liver powder[6] to 1,000 ml. of tap water (McClung and McCoy, 1934). Heat in flowing steam for 1 hour with occasional stirring. Remove from steam and cool almost to room temperature. Dispense in tubes, flasks, or bottles as may be needed. Sterilize for 45 minutes at 15 pounds pressure. The resulting medium, on cooling, should be semisolid with the coarser particles of corn settling to the bottom leaving a 2–3 cm. layer of starchy material at the top.

Use: A useful enrichment medium in studies of anaerobic population of natural samples. (It remains anaerobic throughout prolonged incubation periods). Especially suited for the butyric-butyl group, and recommended for the detection of thermophilic contamination. A very inexpensive and convenient medium suitable for sampling surveys and other studies involving a large number of tubes. Has some diagnostic value, as certain of the butyl groups give a characteristic "head" (a slimy mass of unfermented cellulosic material raised and collected at the top of the liquid) in this medium in contrast to the butyrics which usually do not give this reaction.

2. MEDIA FOR PLATING FOR PURIFICATION

For the pathogenic types a good medium can be made from the liquid obtained by the infusion of beef heart or lean beef tissue, as discussed above, either with or without 0.5% glucose or defibrinated blood or both. Similarly, the butyric-butyl group grow well on a solidified medium prepared from liver broth, with the addition of 0.5% glucose.

Thioglycollate agars. For the pathogenic types Reed and Orr (1941) suggested two other media which may be prepared from dehydrated ingredients which are available commercially. One of these is made by adding 2% agar (for surface colonies) or 0.75% agar (for subsurface colonies) and 0.1% glucose to Brewer's thiogylcollate broth, adjusted to pH 7.6 before sterilization. (The medium

[6]*Dried liver powder.* Difco Laboratories, Detroit, Michigan.

with the smaller percentage of agar is preferred by some for seeded plates as an aid in securing discrete colonies.) An alternate formula is as follows:

Proteose peptone	20 g.	Na_2HPO_4	2 g.
Glucose	1 g.	Sodium thioglycollate	1 g.
Agar	20 g. (or 7.5 g. for subsurface colonies)		
Distilled water	1000 ml.		

Adjust 7.6 pH. If used for subsurface colonies, clarify medium by filtration through paper using reduced pressure.

Use: Satisfactory for routine purification and colony study of pathogenic types. Convenient to prepare, since the ingredients are usually available and for fresh meat extracts are not needed.

Yeast infusion glueose agar. Prepare yeast infusion as follows (although other methods, sometimes preferred, are equally satisfactory): Obtain fresh yeast (starch-free if possible) from a fermentation company and add 10% by weight to several liters of tap water. Autoclave for 3 hours or more. Allow cells to settle by standing for several days at room temperature. Remove liquid infusion by syphon or with the Sharples centrifuge. Sterilize the liquid, after removal from the cells, in screw-capped bottles and store indefinitely. For plating medium add 0.5% glucose and 2.0% agar. Adjust to 7.0 pH; sterilize for 20 minutes at 15 pounds pressure. (*Note*: An equally satisfactory, but considerably more expensive, basal medium may be prepared from dehydrated yeast extract, adding 0.5% yeast extract to distilled water).

Use: Recommended as plating medium for butyric-butyl group.

Peptone-tryptone-glucose agar. If a source of yeast for the preparation of yeast infusion is not readily available, the following plating medium may be substituted which is only slightly less satisfactory than the one above.

Peptone	0.5%	Glucose	0.5%
Tryptone	0.5%	Agar	2.0%

Adjust 7.0 pH before sterilization. (The medium is improved by the addition of 100 ml. of liver infusion, if available).

Use: A satisfactory plating medium for the butyric-butyl group, calling for ingredients which are usually available.

3. MEDIA FOR DETERMINATION OF PHYSIOLOGICAL REACTIONS

Sugar-free Base for Qualitative Fermentative Reactions[7]. Two basal media for use in anaerobic fermentation reactions are given here. Certain general directions are necessary: Indicators should be used to test reaction after incubation or on small samples withdrawn during incubation; they should not be incorporated in the medium, as many anaerobes reduce them to their leuco form. The

[7]Some workers have used a meat infusion broth or other medium which has been rendered sugar-free by fermentation with *Escherichia coli* or *Clostridium perfringens* This seems unnecessary at the present time as most species will grow quite well in one or the other of the media suggested here. If a particular strain should not grow well in the basal medium plus glucose, it is probable that some needed nutrient is not present. For these, as with fastidious aerobes, ascitic fluid may be added, though this will rarely be necessary.

For quantitative studies on fermentation of the sugars the usual problem requires a base medium suitable for the butyric-butyl group. Perhaps the most generally useful basal medium is yeast water infusion prepared according to the method discussed for yeast infusion glucose agar.

following fermentable carbon sources are usually sufficient for differentiation of the common species: lactose, glucose, salicin, sucrose and maltose. The next most useful list includes: mannitol, glycerol, starch, pectin, and cellulose. If there is question concerning the effect of heat on the carbon compound, a concentrated solution may be sterilized by filtration and added aseptically to the basal medium after heat sterilization. In the establishment of the characteristics of new species list the reaction on all the commonly available carbohydrates.[8]

Fermentation Basal Medium of Reed and Orr (1941). Dissolve the following in 1,000 ml. of distilled water:

Peptone or proteose peptone	20 g.	Sodium thioglycollate	1.0 g.
NaCl	5 g.	Agar	1.0 g.
Carbohydrate		10 g.	

Use: Recommended for pathogenic group but not for butyric-butyl group.

Fermentation Basal Medium of Spray (1936). Dissolve the following in 1,000 ml. of distilled water:

Neopeptone	10 g.	Agar	2.5 g.
Tryptone	10 g.	Carbohydrate	10 g.

Adjust to pH 7.3 or 7.4.

Use: Recommended for all types.

Medium for Testing Action on Litmus Milk. This medium is as important with the anaerobes as it is with the aerobes and in fact Spray (1936) used the reactions in this medium as one of the primary characters in his system of classification.

Use either fresh skimmed milk or spray-dried milk powder. In the latter case, mix 90–100 g. of powder with 1000 ml. of distilled water. Prepare a paste with a small amount of water and then dilute this with the remainder of the water. Use the Waring Blendor[9] or other mixing machine if available. Strain through cheesecloth and adjust to pH 6.8. Dispense in tube to which 0.05–0.1 g. of reduced iron[10] is added before the tubing process. If reduced iron is not available, replace the iron powder with a strip of No. 26 gauge black stove-pipe iron. Sterilize by intermittent process or by autoclaving for 15 minutes at 15 pounds. Immediately on removal from autoclave cool the tubes by standing them in cold water. Anaerobic seal is unnecessary as the reduced iron keeps the oxidation-reduction potential at a low level.

---

[8]When interpreting results, make note of the following:

If an organism fails to grow in the basal medium, unless a fermentable carbon source is present, presence of growth indicates ability to ferment the compound in question.

Gas production, *per se*, is not proof of carbohydrate fermentation, as many anaerobic species are highly proteolytic and may produce gas in the cleavage of protein.

[9]Waring Corporation, 1697 Broadway, New York City.

[10]Iron reduced by hydrogen, from Merck Company, Rahway, New Jersey.

Use: Satisfactory for the determination of those characters usually revealed by litmus milk. Of diagnostic aid in the search for *C. perfringens*, due to the fact that this organism gives a stormy fermentation.

Note: This test is not strictly specific for *C. perfringens* as certain non-pathogenic motile species of the butyric-butyl group also give this reaction. They may be separated from *C. perfringens* by virtue of the non-motility of the latter. Robinson and Stovall (1939) recommend the addition of 1.0 ml. of 20% Na_2SO_3 solution and 0.1 ml. of 8% $FeCl_3$ solution to 10 ml. of milk as an additional aid in the diagnosis of *C. perfringens*. This organism produces a blackening reaction.

Medium for Liquefaction of Gelatin. For some species standard nutrient gelatin plus 0.25% glucose may serve as a base medium for testing for liquefaction of gelatin. If the organism in question will grow in such a medium, it is recommended for use. For other species choice may be made between the two formulae which follow:

Gelatin Medium of Reed and Orr (1941). Dissolve the following ingredients in 1,000 ml. of distilled water:

Gelatin	50 g.	Na_2HPO_4	2 g.
Peptone	10 g.	Glucose	1 g.
Sodium thioglycollate	1 g.		

Gelatin Medium of Spray (1936). Dissolve the following ingredients in 1,000 ml. of distilled water:

Difco Nutrient Gelatin	128 g.
Glucose	1 g.

Dissolve gelatin in water taking care not to scorch the gelatin. Include a strip of No. 26 gauge black stove-pipe iron in each tube.

Use: Either of the above media may be used for the pathogenic group. The medium of Spray has the additional advantage of being a presumptive medium for *C. histolyticum* as this organism gives an orange to wine-red color within the first 48 hours of incubation.

Other Media for Testing Proteolytic Action. The action on gelatin represents action on a simple and incomplete protein and positive action is not necessarily an indication that the organism can hydrolyze the complex proteins. The beef heart infusion represents one of the media in which putrefactive action on complex proteins may be recorded. Coagulated serum slants, prepared in the usual manner, inoculated and incubated in an anaerobic jar, represent another type of protein to be tested. Evidence of proteolytic action in this medium is shown by partial or complete liquefaction of the medium. For action on coagulated egg albumin include a small cube of the white of a hard boiled egg in a tube of 1% peptone and 0.2% glucose broth or other liquid medium. Disintegration of this cube during the incubation is evidence of proteolytic action. Peptonization of litmus milk reveals caseinolytic ability. In addition to the above three other media are recommended. It may not be necessary to use all of these but more than one should be included in taxonomic studies because of possible differential reactions.

Alkaline Egg Medium. Mix the yolk of two and the whites of four eggs (preferably in Waring Blendor). Add 1,000 ml. of distilled water and 12 ml. of 1 *N* NaOH. Stir well or mix in Waring Blendor. Add one part of the above to 5 parts of nutrient broth (beef extract

and peptone). Tube in deep columns and autoclave for 20 minutes at 15 pounds. The final medium should be an opaque whitish liquid. Proteolysis is indicated by progressive clearing of the medium.

Brain Medium. Secure fresh sheep (or calf) brains which are as free as possible from injury. Using forceps clean blood and membranous material from brain tissue. Add distilled water, in the ratio of 100 ml. of water to 100 g. of brain, and boil slowly for one half hour. Put brains through potato ricer. Add 1.0% peptone and 0.1% glucose to the resulting mixture and heat slightly to put peptone in solution. Tube in deep columns while the mixture is stirred in order to effect an even distribution of the brain tissue. Reduced iron or a strip of black stove-pipe iron or iron wire may be added to the tube before tubing the liquid mixture. Sterilize in autoclave for 30 minutes at 15 pounds and *check sterility* by incubation at 37° C. for a minimum of 24 hours. The finished medium has approximately an equal amount of liquid broth above the brain particles. Proteolysis is indicated by putrefactive odors, a disintegration of the particles and a blackening reaction.

Use: The blackening reaction of this medium has some diagnostic significance (Hall and Peterson, 1924). This medium is also valuable for many species for the production of spores and hence as a stock culture medium.

Milk Agar for Testing Proteolytic Action. Reed and Orr (1941) suggest the following medium: Mix equal parts of skim milk (reconstituted from powder) and a plating agar (see their media in section on plating media for purification). Autoclave the two media separately and mix just before pouring. Proteolysis is indicated by a wide clear zone surrounding the growth.

Medium for Production of H_2S. Probably most, if not all, species of anaerobes produce H_2S, at least in trace amounts. From the discussion of McCoy, *et al.* (1926), Spray (1936), Pacheco e Costa (1940) and Reed and Orr (1941), we conclude that there is, as yet, no standard medium for this reaction. The media listed below were found to be satisfactory by Reed and Orr (1941); and it is recommended that the exact method of preparation be listed in published reports for any additional medium which may be devised.

Medium 1

Proteose peptone	20 g.	Glucose	1 g.
Na_2HPO_4	2 g.	Agar	2 g.
Water	1000 ml.		

Dissolve ingredients, adjust to pH 7.6, and add 10 ml. of 2% lead acetate. This results in a cloudy precipitate which, however, remains after autoclaving in a reasonably stable suspension.

Medium 2

Proteose peptone	20 g.	Glucose	1 g.
Na_2HPO_4	2 g.	Water	1000 ml.

Dissolve ingredients, adjust to pH 7.6, and add 10 ml. of a 1.5% bismuth and ammonium citrate solution. This ordinarily produces a solution which remains clear after autoclaving.

Medium for the Formation of Indole and Skatole. The following medium will usually be found satisfactory:

Tryptone (Bacto)	20 g.	Sodium thioglycollate (for pathogenic group only)	1 g.
Na_2HPO_4	2 g.	Agar	1 g.
Glucose	1 g.		
Water	1000 ml.		

Application of test (see Roessler and McClung, 1943): Place 2 drops of the culture (withdrawn by pipette) in a spot plate; add 2 drops of vanillin (5% in 95% ethyl alcohol) and then 3 drops of concentrated HCl. The addition of one drop of 0.1% $NaNO_2$ causes the violet-pink of skatole to become dark purple but the orange color characteristic of indole is not changed.

Medium for Nitrate Reduction. (See Reed, 1942). As certain species reduce nitrites as well as nitrates, there should be included a test for the presence (or disappearance) of nitrates as well as the appearance of nitrites. A negative nitrite test is of no significance. The medium of Reed and Orr (1941) is satisfactory:

Tryptone (Bacto)	20 g.	Agar	1 g.
Na_2HPO_4	2 g.	KNO_3	1 g.
Glucose	1 g.	Water	1000 ml.

Adjust pH to 7.6 before autoclaving.

4. OTHER MEDIA OF VALUE

Medium for demonstration of capsules and spores. It is sometimes inconvenient to use animal autopsy material for demonstration of capsules. Svec and McCoy (in press) recommend the following medium for demonstration of capsules and spores of *C. perfringens.* Presumably it will be suitable for other species.

Casein hydrolysate (acid)	35 ml.	K_2HPO_4	5 g.
Ovalbumin hydrolysate (acid)	15 ml.	Sodium thioglycollate	1 g.
Yeast water (prepared by autoclaving 20% wet weight of yeast in water)	100 ml.	$(NH_4)_2SO_4$	2 g.
		Tryptophane	12 mg.
		Glucose	2.5 g.
Sodium lactate	5 ml.	Distilled water to make	1000 ml.

Adjust pH to 7.4 and sterilize 25 minutes at 15 pounds.

To prepare acid hydrolysates: Autoclave 200 g. casein (or egg albumin), 110 ml. concentrated HCl and 170 ml. distilled water for 45 minutes at 12 pounds. If desired, decolorize with norite.

Medium for spore production by butyric-butyl group. If cultures of this group do not sporulate readily on plain corn mash (prepared according to directions for corn-liver medium except that the liver powder is omitted), use potato infusion prepared as follows:

Irish potatoes	200 g.	$(NH_4)_2SO_4$	1 g.
Glucose	5 g.	$CaCO_3$	3 g.
Tap water to make	1000 ml.		

Peel potatoes and add water. Steam for one half hour or boil slowly until soft and put through potato ricer. Add other ingredients and bring up to original volume. Cool and tube, with stirring, so as to obtain an even distribution of the potato particles.

Medium for toxin production. In Leaflet III there is mention of the fact that beef heart infusion or glucose meat infusion is satisfactory for toxin production by most toxigenic species. Another medium, proposed by Reed, Orr, and Baker (1939), may be recommended for the gangrene group. This is prepared from commercially available ingredients as follows:

NaCl	2.0 g.	Gelatin, Difco	50.0 g.
$MgSO_4$	0.02 g.	Peptone, Bacto	10.0 g.
Na_2HPO_4	5.76 g.	Glucose	2.0 g.
KH_2PO_4	0.24 g.	Water	1000 ml.

Adjust to pH 7.7 and autoclave at 15 pounds.

REFERENCES

American Public Health Association. 1936. Standard methods of Water Analysis. Eighth edition. Published by the Association, New York, 1936.

Anderson, J. S., Happloid, F. C., McLeod, J. W. and Thomson, J. G. 1931. On the existence of two forms of diphtheria bacillus—*B. diptheriae gravis* and *B. diphtheriae mitis*—and a new medium for their differentiation and for the bacteriological diagnosis of diphtheria. J. Path. & Bact., **34**, 667–81.

Ayers, S. H., Rupp, P., and Johnson, W. T. 1919. A study of the alkali-forming bacteria in milk. U. S. Dept. Agric., Bul. 782.

Bailey, S. F., and Lacy, G. R. 1927. A modification of the Kligler lead acetate medium. J. Bact., **13**, 183–9.

Blubaugh, L. V., and Reed, W. 1943 Sodium thioglycollate as an antibacteriostatic agent. Its use in sterility testing. J. Bact., **45**, 44.

Brewer, J. H. 1940a. A clear liquid medium for the "aerobic" cultivation of anaerobes. J. Bact., **39**, 10.

Brewer, J. H. 1940b. Clear liquid mediums for the "aerobic" cultivation of anaerobes. J. Amer. Med. Assoc., **115**, 598–600.

Clark, W. M. 1928. The Determination of Hydrogen-ions. Third edition. Williams and Wilkins, Baltimore.

Cohen, Barnett. 1922. Brom cresol green, a sulfonphthalein substitute for methyl red. Proc. Soc. Exp. Biol. Med., **20**, 124.

Cohen, Barnett. 1927. Synthesis and indicator properties of some new sulfonphthaleins. Public Health Repts., **41**, 3051.

Cole, S. W. and Onslow, H. 1916. A substitute for peptone and a standard nutrient medium for bacteriological purposes. Lancet, 1916, II, 9–11.

Daily, F. K., and Blubaugh, L. V. 1941. The elimination of bacteriostatic action by the use of sodium thioglycollate medium. J. Bact., **42**, 147–148.

Douglas, S. R. 1922. A new medium for the isolation of *B. diphtheriae*. Brit. J. Exp. Path., **3**, 263–7.

Federal Register. 1942. **7**, No. 26, 781–2.

Hall, I. C. 1921. Criteria in anaerobic fermentation tests. J. Inf. Dis., **29**, 321–43.

Hall, I. C. and Peterson, E. 1924. The discoloration of brain medium by anaerobic bacteria. J. Bact., **9**, 211–24.

Hewitt, L. F. 1937. Oxidation-reduction potentials in bacteriology and biochemistry. 4th edition. London County Council.

Hitchens, A. P. 1921. Advantages of culture mediums containing small percentages of agar. J. Inf. Dis., **29**, 390–407.

Huntoon, F. M. 1918. "Hormone" medium; a simple medium employable as a substitute for serum medium. J. Inf. Dis., **23**, 169–72.

Knight, B. C. J. G. 1931. Oxidation-reduction potential measurement in cultures and culture media. Chapter XIII (pp. 165–73) *in* Vol. IX *of* System of Bacteriology. (Gt. Brit.) Med. Research Council.

Kracke, R. and Teasley, H. E. 1930. The efficiency of blood cultures. J. Lab. & Clin. Med., **16**, 169.

LIEFSON, EINAR. 1935. New culture media based on sodium desoxycholate for the isolation of intestinal pathogens and for the enumeration of colon bacilli in milk and water. J. Path. & Bact., **40**, 581–99.

LEVINE, MAX, and SCHOENLEIN, H. W. 1930. A Compilation of Culture Media for the Cultivation of Microorganisms. Williams and Wilkins, Baltimore.

MCCLUNG, L. S. 1940. The use of dehydrated thioglycollate medium in the enrichment of spore-forming anaerobic bacteria. J. Bact., **40**, 645–8.

MCCLUNG, L. S. 1943. Thioglycollate media for the cultivation of pathogenic clostridia. J. Bact., **45**, 58.

MCCLUNG, L. S., and MCCOY, E. 1934. Studies on anaerobic bacteria. I. A corn-liver medium for the detection and dilution counts of various anaerobes. J. Bact., **28**, 267–77.

MCCOY, E., FRED, E. B., PETERSON, W. H., and HASTINGS, E. G. 1926. A cultural study of the acetone butyl alcohol organism. J. Inf. Dis., **39**, 457–83.

MARSHALL, M. S., GUNNISON, J. B., and LUXEN, M. P. 1940. Test for the sterility of biologic products. Soc. Expt. Biol. and Med., Proc., **44**, 672–3.

NUNGESTER, W. J., HOOD, M. N., and WARREN, M. K. 1943. The use of thioglycollate media for testing disinfectants. J. Bact., **45**, 44.

PACHECO, G., E COSTA, G. A. 1940. Produçao de H_2S pelos clostridios anaerobios. Mem. Inst. Oswaldo Cruz, **35**, 311–6.

REED, G. B., and ORR, J. H. 1941. Rapid identification of gas gangrene anaerobes. War Med., **1**, 493–510.

REED, G. B., and ORR, J. H. 1943. Cultivation of anaerobes and oxidation-reduction potentials. J. Bact., **45**, 309–20.

REED, G. B., ORR, J. H., and BAKER, M. C. 1939. Gas-gangrene-toxin production. Soc. Expt. Biol. and Med., Proc., **42**, 620–1.

REED, R. W. 1942. Nitrate, nitrite and indole reactions of gas gangrene anaerobes. J. Bact., **44**, 425–31.

ROBINSON, C. L., and STOVALL, W. D. 1939. A clinical bacteriological test for the recognition of *C. welchii* in wounds. Amer. Jour. Clin. Path., Technical supplement, **9**, 27–32.

ROESSLER, W. G., and MCCLUNG, L. S. 1943. Suggested method for use of vanillin as a test reagent for indole and skatole production by bacteria. J. Bact., **45**, 413.

SPRAY, R. S. 1936. Semisolid media for cultivation and identification of the sporulating anaerobes. J. Bact., **32**, 135–55.

SVEC, M. H., and MCCOY, E. In Press. A chemical and immunological study of the capsular polysaccharide of *Clostridium perfringens*. J. Bact., in press.

TITTSLER, R. P. and SANDHOLZER, L. A. 1936. The use of semi-solid agar for the detection of bacterial motility. J. Bact., **31**, 575–80.

WILSON, W. J. 1923. Reduction of sulphites by certain bacteria in media containing a fermentable carbohydrate and metallic salts. J. Hyg., **21**, 392–8.

WILSON, W. J. and BLAIR, E. M. M'V. 1926. A combination of bismuth and sodium sulphite affording an enrichment and selective medium for the typhoid-paratyphoid groups of bacteria. J. Path. and Bact., **29**, 310–1.

WINOGRADSKY, S. and OMELIANSKI, V. 1899. Ueber den Einfluss der organische Substanzen auf die Arbeit der nitrifzierenden Mikroben. Centbl. f. Bakt. II Abt., **5**, 329–43, 377–87, 429–40.

LEAFLET III

THE STUDY OF OBLIGATELY ANAEROBIC BACTERIA

Prepared by
COMMITTEEMAN ON ANAEROBIC METHODS

4th EDITION
NOVEMBER, 1943

Note—The first edition of this leaflet was written, and the second edition revised, by Ivan C. Hall. The third edition, prepared by J. Howard Brown, represented revision of certain sections of the second edition. This edition has been prepared for the Committee by L. S. McClung.

LEAFLET III

THE STUDY OF OBLIGATELY ANAEROBIC BACTERIA[1]

It is impossible to list here all of the methods which have been proposed for the study of anaerobic bacteria; an attempt is made, however, to outline a number of technics which have been used widely and which should ordinarily be suitable for routine studies of anaerobic species. Those interested in other technics are advised to consult Section B of the subject index bibliography relating to the anaerobic bacteria (McCoy and McClung, 1939; McClung and McCoy, 1941). The worker who has had no experience with anaerobic bacteria should study some of the articles which deal with principles of anaerobic culture or which record the results of a study of a considerable number of strains (Committee upon anaerobic bacteria and infections, 1919; Fildes, 1931; Hall, 1922, 1928, 1929; Heller, 1921; Knorr, 1923, 1924; McCoy, *et al.*, 1926, 1930; McIntosh, 1917; Meyer, 1928; Reed and Orr, 1941; Robertson and O'Brien, 1929; Soule, 1932; Spray, 1936; Zeissler, 1930; Zeissler and Rassfeld, 1928). These are suggested rather than the monographs (Hibler, 1908; Weinberg et Séguin, 1918; Weinberg et Ginsbourg, 1927; Weinberg, Nativelle, et Prévot, 1937) which are not distributed widely.

The organisms which we call obligate anaerobes, are those that require strict exclusion of atmospheric oxygen from the immediate environment in which they are to grow. It is not easy to answer the question of the best method of determining whether or not a given organism is an obligate anaerobe. The catalase reaction, when applied to pure culture, gives presumptive evidence, for obligate anaerobes usually are catalase-negative. For this reaction a plate culture of the organism in question is flooded with a 10% solution of H_2O_2. The evolution of gas bubbles from the colonies denotes the presence of catalase.

If the proper material for the catalase reaction is not available,

[1]The methods and technics suggested herein are those recommended for use with the more common spore-forming anaerobic species. Many of these methods are suitable, also, for the study of the non-spore-forming types, and for the present no attempt will be made in this Leaflet to outline particular methods of study for these. If the technics herein outlined do not prove satisfactory, the worker interested in the pathogenic non-spore-formers should consult the review of Dack (1940) and the publications of Prévot (1924, 1925, 1938, 1940a, 1940b). Non-pathogenic types exist, as for example, the methane organisms discussed by Barker (1936). For the complete literature on all types refer to Section Id (non-spore-formers) in the bibliography of McCoy and McClung (1939) and McClung and McCoy (1941).

or in case of doubt, the following technic will usually suffice to characterize an anaerobic strain and to differentiate if from the aerobes: Inoculate, while the agar is molten, several deep tubes (8–9 cm. columns of medium) of a suitable nutrient agar medium (see Leaflet II) containing 1.0% glucose; allow these to solidify in an upright position, and incubate the tubes at several temperatures or at the optimum temperature for the organism in question; adjust the seeding so that relatively few (e.g., 25–50) colonies per tube will result. With an obligate anaerobe, all of the colonies should be localized in the bottom of the tube and none should appear on the surface or in the upper 1 cm. layer. Likewise, with pathogenic organisms cultured in fluid thioglycollate medium, the growth should be confined to the lower section of the medium and no growth should result in the upper layer wherein the methylene blue is recolorized. If growth does occur in the upper layer of either medium, the culture is either not an obligate anaerobe or is contaminated with an aerobic or a facultative species.

ANAEROBIC CULTURE METHODS AND EQUIPMENT

All of the procedures which have been devised for the cultivation of anaerobic bacteria have the single purpose of excluding *atmospheric* oxygen from the environment in which the growth is to take place. With certain tubed media the oxygen potential may be reduced sufficiently by constituents of the medium to permit anaerobic growth (Hewitt, 1937; Knight, 1931; and Reed and Orr, 1943). Since, however, this is rarely possible for surface cultures on a solid medium, usually plate and slant cultures are incubated within a closed container from which the oxygen is removed by one or another means. A study of the various methods shows that no single procedure may be proposed as the *best* technic but that the method of choice will depend upon the prevailing circumstances. A procedure which is ideal for one situation may be impractical or impossible to apply with other conditions. Each of the technics outlined below is recommended within the limits proposed in the discussions.

Use of Methylene Blue as Indicator of Anaerobiosis. For all types of anaerobic jars and containers, except individual plating or tube culture systems, it is convenient to include an indicator tube which will serve as a check on the development of anaerobiosis. The most commonly used system utilizes the change of methylene blue from the colored (oxidized state) to the leuco form (reduced state) Using the solution prepared as given below, any system which gives sufficient degree of removal of oxygen from the atmosphere for anaerobic growth to develop will cause the blue color of the solution to disappear or will maintain the colorless condition if the solution is

boiled (heat reduction) immediately prior to its being placed in the container. A somewhat less sensitive system can, in an emergency, be prepared by adding a tinge of color from Loeffler's alkaline methylene blue to a tube of glucose broth.

The procedure recommended (Fildes, 1931) is: Prepare three stock solutions: (1) 6.0 ml. N/10 NaOH diluted to 100 ml. with distilled water; (2) 3.0 ml. 0.5% aqueous methylene blue diluted to 100 ml. with distilled water; (3) 6.0 g. of glucose in 100 ml. distilled water to which has been added a small crystal of thymol.

Each time the indicator solution is needed, mix equal parts of the three solutions in a test tube and boil in a cup of water until the color disappears. Place tube in anaerobic container immediately and begin process of securing anaerobic conditions. If the container is satisfactorily deoxygenated, the color in the solution should not reappear. If the blue color does return it is a sign that the container leaks or has not been satisfactorily exhausted of oxygen. (In the vegetable tissue jar, to be described, the color may appear but will disappear with the development of anaerobiosis during the incubation period).

Biological Methods for Oxygen Removal

VEGETABLE TISSUE JAR

Materials for method of McClung, McCoy and Fred (1935): (1) Jar, or other container which may be sealed air tight (*Recommended:* 6″ × 18″ or 6″ × 12″ Pyrex cylinder[2]); (2) square (7″ × 7″) of plate glass or a glazed plate; (3) plasticene[3], ¼ pound; (4) glass tumbler; (5) supply of oats or other grain (other tissues, particularly chopped Irish potatoes, may be used, but are less conveniently stored for occasional use, and in some cases produce objectionable odors which are evident when the jar is opened); (6) tap water.

Method: Place inverted tumbler (if plates are to be used), or other support, in bottom of cylinder. Add oats to fill at least one tenth of the capacity of the cylinder. Add sufficient tap water to moisten the oats. Stack plates (or other cultures) on support. Add tube of methylene blue solution (see above). Place layer of plasticene (previously softened by placing in incubator) on rim of cylinder. Push plate glass square firmly against plasticene; using fingers, press the clay against both the square and the cylinder until a satisfactory seal is obtained. Place jar in incubator immediately. (A 40–48-hour incubation period is recommended).

If plate cultures are employed, use unglazed porcelain ("clay") tops[4] to replace the ordinary petri dish cover to absorb the moisture which collects within the cylinder. If porcelain tops are unavailable, add a petri dish lid containing $CaCl_2$ to absorb the moisture.

---

[2]*Pyrex cylinder.* Corning Glass Works, Corning, New York or supply house. Pyrex Catalogue No. 850.

[3]*Plasticene* The most satisfactory product of this type seems to be the English clay called "Plasticene" (gray or green colored). This is obtainable in this country from J. L. Hammet Company, Cambridge, Massachusetts, and perhaps other supply houses. Other types may be found which are satisfactory but these must be tested individually for suitability as some have been encountered which dry to a hard cake upon incubation.

[4]*Unglazed porcelain ("clay") tops for Petri dishes.* The Coors porcelain dish, sold by Arthur H. Thomas Company, has been found to be more uniform in size and quality than others tested.

Advantages: The method is inexpensive and employs easily available materials. No special apparatus is required—an advantage in laboratories where anaerobic cultures are not usually prepared. It may be used at any incubation temperature without danger of explosion. It is particularly suitable in problems requiring large numbers of plate cultures. It is recommended especially for cultural and physiological studies of strains which have been purified by other methods. *Disadvantages:* Several hours may be needed for anaerobic conditions to become established and therefore the method is not suitable when the results are required quickly. It is not recommended for routine clinical use where speed of isolation of pure culture is an important factor. With certain enrichments it is not suitable for purification of species contaminated with aerobic spore-forming bacteria due to the quick growth of these forms. In plate culture experiments, as in the isolation of new strains, no one plate may be removed from the cylinder for observation until the end of the incubation period, for to do so would destroy the anaerobic conditions within the cylinder.

USE OF AEROBE TO ABSORB OXYGEN

Another biological method for oxygen removal utilizes the growth of an aerobic organism (usually *Staphylococcus aureus*, *Serratia marcescens*, or *Saccharomyces cerevisiae*). A wide variety of applications of this system have appeared in the literature. The technics suggested[5] below involve the growth of the aerobic organism in pure culture on a medium separate from that on which the anaerobe is to be cultured.

Method A

Materials for method of Snieszko, 1930: (1) Two petri dishes of ordinary size; (2) paper tape, scotch tape, adhesive plaster, or plasticene; (3) culture of *Serratia marcescens* or other fast growing aerobic organism; (4) tube of nutrient agar.

Method: Select two petri dishes which have bottoms of exactly the same size and sterilize these in position in their usual top sections. Pour nutrient agar into the bottom half of plate A, and after solidification, streak the medium heavily (or flood across surface with 0.5 ml. of broth culture) with the aerobic organism. (As an alternate method, seed the agar before pouring.) Pour into plate B, a medium suitable for the anaerobe (see Leaflet II, 9th Ed.); when hard streak with the sample or culture of the anaerobe (or seed with the latter prior to pouring).

Remove the two bottoms from their respective tops and fit together at their rims. Use tape or other sealing device around the juncture to provide an air-tight seal. Place plate in the incubator immediately. If thermophilic anaerobic cultures are to be made, replace the *S. marcescens* by a thermophilic aerobe, or before placing plates in thermophilic incubator, incubate for 18 hours at 32° C. to allow *S. marcescens* to grow and to use the oxygen.

Advantages: No elaborate equipment is needed, since the method uses ordinary petri plates and other common materials. Thus it is available as an emergency

[5]These are similar to the Fortner method and are recommended in place of it. In the Fortner method the aerobe is streaked on one half of the plate and the anaerobe on the other half of the same dish.

method in almost any laboratory at any time. The technic is so simple that no previous experience with the method is necessary for success. Since each set of plates is an individual unit, observation of the growth of the anaerobe may be made at any time without destroying the anaerobic conditions. *Disadvantages:* It is somewhat time-consuming when large numbers of platings are to be made, and, therefore, not suitable in laboratories where routine plating of a number of cultures is not an unusual event. Anaerobic conditions may not be attained sufficiently quickly to prevent death of the inoculum of non-spore-forming species or vegetative cells of anaerobic spore formers.

Method B

Materials for method similar to that of Marshall and Nordby (1942): (1) One petri plate of usual size (bottom should be 15 mm. deep); (2) one small petri plate[6] (75 mm. × 10 mm.); (3) culture of *S. marcescens;* (4) tube of nutrient agar.

Method: Pour nutrient agar in bottom half of the regular size plate, and streak or flood surface with aerobe. Pour agar for anaerobe in bottom half of small plate. Remove this bottom from its top and press down in agar of the regular size dish.

Advantages: A simple method suitable for small numbers of plates. The purchase of the small-sized plates is less expensive than some of the more elaborate apparatus required by certain other methods. *Disadvantages:* Necessity of purchase of the small-sized plates.

Chemical Methods for Oxygen Removal

Many of the methods proposed for removal of oxygen from the environment for anaerobic culture involve the initiation of a chemical reaction in which oxygen is consumed. Of the various systems which have been suggested, those which are recommended have been tested and used sufficiently to show their utility and do not require elaborate apparatus.

PHOSPHORUS JAR

Materials: (1) Sticks of yellow (or white) phosphorus (which *must be kept under water in tightly stoppered wide mouth bottle;* the small sticks, $\frac{5}{16}$ inch diameter, are the most useful); (2) Pyrex cylinder or any convenient jar or container which may be sealed air tight; (3) pair of long forceps or chemical tongs; (4) plasticene; (5) small amount of tap water.

Method: Place small amount of tap water in bottom of cylinder to remove the P_2O_5 which forms. Stack inoculated plates or tubes on support. Add tube of methylene blue solution (see p. III$_{13}$-5). Place small (50 ml.) beaker on top of cultures. Remove two or three short (1½ to 2 inch) pieces of phosphorus from water *with forceps or tongs* and place in beaker. Immediately put lid on jar and seal with plasticene. (Upon drying for a few minutes, the phosphorus should ignite spontaneously and remain burning as long as there is oxygen present). If experience shows that the

[6] *Small petri plates.* Central Scientific Company, Chicago, Illinois.

phosphorus used does not ignite spontaneously but merely gives off a grey smoke, ignite it before the jar is sealed by a match *held with the forceps.* Since considerable heat is developed, place beaker, unless resistant glass is used, three inches from the top of the container and put a "blank" plate under the beaker rather than an inoculated plate. After the phosphorus ignites, and the jar is tightly sealed, place it directly in the incubator. At the time the container is opened, have available a crock or pan filled with water. As soon as the lid is taken from the jar, remove the beaker containing the phosphorus with the tongs and submerge under the water in the pan and save for later use. After this, remove the cultures from the jar.

Advantages: Quick method of obtaining anaerobiosis. It is relatively inexpensive since the only materials are phosphorus and a container which may be sealed. *Disadvantages:* Care must be exercised to prevent accidental burns which are very painful. Inexperienced technicians should be cautioned concerning the dangers.

ALKALINE PYROGALLOL METHODS

Another chemical method for removing oxygen in order to promote anaerobic growth is to utilize the oxygen absorptive capacity of the reaction between alkali and pyrogallic acid. Of the technics and devices reported which make use of this reaction, two may be recommended as being especially useful. One of these concerns a technic applied to individual plate culture and the other relates to a system for individual tube cultures.

Spray (or Bray) Plate Cultures

Materials: (1) Spray (1930) anaerobic dish[7]; (2) plasticene (see footnote 3) or tape for sealing; (3) 20% aqueous NaOH. (4) 40% aqueous pyrogallic acid.

Note: The Spray dish consists of an ordinary glass petri dish top and a special bottom which is deep and which has a raised ridge across the center. The top of the bottom dish has a lip into which the top section of the dish fits. Although constructed of heat resistant glass, in practice considerable breakage during sterilization and handling of the Spray dish may be encountered. This is eliminated in the Bray[8] dish, which is Pyrex, and which is essentially the same in design as the Spray dish. In the Bray dish, however, the need for the lip is eliminated since the top of the bottom section is slightly smaller in diameter than the remainder of the bottom section. This allows the top to fit down over the rim of the bottom section.

Method: Pour anaerobic medium in the top half of the dish, and after solidification, streak from sample or culture, or pour seeded plate. After inverting dish, place 10 ml. of 20% aqueous NaOH solution in one section of the bottom dish and 4 ml. of 40% aqueous pyrogallic acid in the other. Seal dish with plasticene or tape. Tilt dish to mix solutions and place in incubator.

[7]*Spray anaerobic dish.* Fisher Scientific Company, Pittsburgh, Pennsylvania, or E. H. Sargent Company, Chicago, Illinois.

[8]*Bray anaerobic dish.* Corning Glass Works, Corning, New York, Pyrex No. 3155, or dealer.

Advantages: Anaerobiosis is attained quickly. It is a useful method for single plate culture. Since each plate is a single unit, observations may be made at any time and any particular plate of a series may be opened when visual inspection reveals growth to be at the desired stage. Recommended for clinical laboratory technicians seeking a quick method of purification of possible pathogenic types. *Disadvantages:* Some time is required to prepare the individual dishes; therefore laboratories doing a great deal of routine work may desire to use instead some of the anaerobic jars. Special plates must be purchased.

Tube Culture

Method A

Materials: (1) Agar slant of suitable anaerobic medium; (2) pyrogallic acid crystals; (3) 10% aqueous NaOH; (4) rubber stopper.

Method: Inoculate agar slant with anaerobic organism or from sample to be cultured. Flame mouth of tube before replacing plug. Cut off the end of the cotton plug which extends beyond the mouth of the tube and push the remaining portion into the tube for a distance of about 2 cm. Fill this space with pyrogallic acid crystals and pour 2 ml. of 10% NaOH upon the crystals. *Immediately* insert rubber stopper and invert tube in such a fashion that the water of condensation does not run across the slant. Incubate tube in inverted position.

Method B

Materials for method of Griffin (1932): (1) Two test tubes with approximately $\frac{5}{8}$ inch diameter (one empty and the other containing a liquid or slant culture of the anaerobe); (2) two one-holed rubber stoppers to fit tubes; (3) short piece of small diameter rubber tubing; (4) two short pieces of glass tubing of diameter to fit tightly in holes of rubber stoppers; (5) small glass vial; (6) dry pyrogallic acid; (7) strong aqueous NaOH.

Method: Put a column of pyrogallic acid, approximately 1½ inches high, in the bottom of the empty tube. Stand empty vial in this acid. With pipette, fill vial two thirds full of NaOH solution. Fashion a connecting unit from the rubber stoppers, and rubber and glass tubing. Insert one of the stoppers in the tube with the chemicals. Push down cotton plug in culture tube to a level one inch above the medium. Insert second stopper in this tube. Tilt tube containing chemicals sufficiently to allow NaOH solution to spill over the acid.

Advantages: Good method for single tube culture. If a supply of chemicals is at hand, it is useful as an emergency system, when the special equipment required by other systems is not available. *Disadvantages:* Not suitable for large numbers of cultures, or, at least, such use would be more time consuming than other methods.

CHROMIUM AND SULFURIC ACID METHOD

Rosenthal (1937) introduced a new system for creating an anaerobic environment using the reaction of H_2SO_4 on powdered chromium to release hydrogen. This flushes out the oxygen by replacing the

air normally present within the container. The method has been modified by Mueller and Miller (1941) and their report forms the basis of the description below.

Materials: (1) A suitable container (see below); (2) fruit jar rubber ring moistened with glycerol or plasticene; (3) chromium powder[9]; (4) H_2SO_4, 15% by volume (3 vol. conc. acid to 17 vol. distilled water); (5) Na_2CO_3.

Note: In this method a desiccator equipped with a stopcock may be used if available; or, for tube cultures a 2-quart Ball fruit jar, prepared as follows, can be recommended: Have a metal casting of the glass cap made. Solder a short length of brass tubing into a hole drilled through the cap. Attach a short U-tube of 5 or 7 mm. glass tubing by a rubber connection. Dip the other end of the U-tube below the surface of mercury (about 2 ml.) in the bottom of a small tube about 2 inches in length. Plug the open end of this tube with cotton to prevent spattering of the mercury. Tie this latter tube to the brass tubing or hold in place by a rubber band.

Method: Place inoculated tubes in jar. Add tube of methylene blue solution (see p. III43-5). Add 3 g. of chromium powder and 1 g. of Na_2CO_3. Using a funnel, introduce 30 ml. of 15% H_2SO_4. Clamp lid on jar immediately; if plasticene is used, prepare the seal around the lid, and allow the hydrogen and CO_2 to escape through the mercury trap tube. As soon as the bubbling subsides, place the jar in the incubator.

Advantages: Quick method of obtaining anaerobiosis for tube culture. With other containers the system may be used for plate cultures. Relatively inexpensive chemicals are employed, though the powdered chromium may not always be available. *Disadvantages:* Necessity of securing metal castings of jar top. Outsides of tubes become covered with chemicals necessitating rinsing when they are removed from container for examination.

Oxygen Removal by Combustion Using Laidlaw Principle

For laboratories which are engaged in problems where anaerobic plating is to be done frequently, it is advisable to plan for this and to purchase equipment accordingly. Although the systems discussed above may be adequate for this purpose, it is well to consider one of the jars which utilize, on the Laidlaw (1915) principle, combustion as a means of securing the anaerobic environment. These methods were designed especially for incubation of plates, but other culture vessels (flasks, tubes, bottles, etc.) may be used. Jars using this principle are those of Brewer (Brown and Brewer, 1938) and McIntosh and Fildes (Fildes and McIntosh, 1921).

BREWER ANAEROBIC JAR[10]

Materials for method of Brown and Brewer (1938): (1) *Brewer jar* complete with electric cord; (2) source of illuminating gas or

[9] *Chromium powder*—98% pure; e.g., from Fisher Scientific Company, Pittsburgh, Pennsylvania or Eimer and Amend, New York, New York.

[10] *Brewer jar.* Baltimore Biological Laboratory, Baltimore, Maryland and Fisher Scientific Company, Pittsburgh, Pennsylvania.

hydrogen; (3) tube of soda lime; (4) plasticene (see footnote 3); (5) water vacuum pump for evacuation.

Method: Place plates in Brewer jar. Add tube of methylene blue solution (see p. 111_{13}-5). Include a tube of soda lime in the jar to absorb excess CO_2. Place roll of (warmed) plasticene around rim of jar. Put on lid and press down on plasticene to form seal. Add the lid clamp but tighten *only slightly. If used with illuminating gas*, attach the jar by the rubber tubing to the water vacuum pump. Evacuate until the manometer or gauge reads approximately 20 cm. or 8 inches. After this degree of evacuation is reached, connect the rubber tube to the gas supply (a three way stop-cock facilitates this change without loss of vacuum). Attach the electric plug (110 volt AC or DC) and allow the gas and electric current to remain attached for 30 to 45 minutes. At the end of this time clamp the rubber tube tightly, remove the electric cord, and place the jar in the incubator. (Formation of water droplets on the inside walls of the jar indicates the proper functioning of the apparatus.) To open the jar, remove the clamp and insert a knife blade between the lid and rim of the jar. *If used with hydrogen*, attach the jar, without evacuation, to the hydrogen tank and admit the gas at a pressure of 1–2 lb. per square inch. Attach the electric connection and allow the current and gas both to remain on for 30 minutes. Then treat the jar as above.

Advantages: Convenient system for incubation of a number of plates in experiments where speed of obtaining anaerobiosis is essential. Recommended for clinical laboratories. Inexpensive system after the initial outlay for apparatus. Danger of explosions is less in the Brewer jar than in the McIntosh-Fildes jar. *Disadvantages:* Some possibility of explosion or cracking of jar. Initial expense of equipment is more than for other methods discussed above — but this may be a good investment if routine work is to be done over a period of time. Requires source of hydrogen or illuminating gas and electricity; while these are available in most laboratories, they are not available in others such as some mobile laboratory units, temporary laboratories in field surveys, etc.

McINTOSH AND FILDES JAR[11]

Materials: (1) McIntosh and Fildes jar; (2) protective box or cage of galvanized wire; (3) cylinder of hydrogen (*preferable*) or hydrogen generator; (4) reducing valve for hydrogen cylinder; (5) resistance coil (approximately 175 ohms for 110 volts or 350 ohms for 220 volts); (6) electrical wire for connections; (7) three-foot length of rubber tubing.

Method: (Adapted from various sources, including directions issued with jar purchased from Arthur H. Thomas Company): Clean surfaces of jar and lid with xylol. Apply suitable sealing medium or hard tallow to these. Grease tips and threads of needle valves. Place cultures in jar and add tube of methylene blue indicator solution (see p. 111_{13}-5). Place lid on jar and tighten the

[11] *McIntosh and Fildes jar.* Arthur H. Thomas Company, Philadelphia, Pennsylvania. Model No. 1085 (glass) or 1085-B (aluminum). A convenient cage is Model No. 1085-F.

large milled head sufficiently to make the lid gas-tight but not to the point at which the action of the coiled spring is ineffective. Tighten the lock nut (the smaller and concentric milled head). Introduce hydrogen from cylinder, through reducing valve set for two pounds, and keep flowing for two minutes or more. Test whether or not all the air has been removed by attaching a rubber hose to the exit valve and allowing the gas to escape in a cup of soapy water. If the gas bubbles fail to "explode" when a lighted match is applied but ignite to burn with a non-luminous flame, the concentration of hydrogen is sufficient to proceed. Close both valves and connect the wiring terminals to an electric source of correct voltage *and through a 0.6–0.7 ampere resistance.* Formation of droplets of water on the inside walls of the jar indicates correct functioning of the apparatus. After a negative pressure develops (a few minutes) add more hydrogen slowly. Continue the current for 30 minutes. Then tighten the valves of the jar and remove the electric connection.

Advantages and disadvantages: See above for Brewer jar. Apparently there is greater danger of explosions with the McIntosh and Fildes jar than with the Brewer jar. Inexperienced technicians are warned to proceed with caution when using this apparatus.

Plating System Using Strongly Reducing Medium

Recently there has been introduced by Brewer (1942) another single plating device which has much to recommend it. Because of its promise it is introduced here even though it has not as yet been used sufficiently widely to establish a reputation. The dish must be used with an agar containing highly reducing agents. The design of the dish is such that the top of the dish rests, at its periphery, on the medium to form a seal, and the remainder of the dish is slightly raised. Thus only a small amount of air is trapped over the surface of the agar and this is removed by means of the reducing action of the medium.

BREWER CULTURE DISH[12]

Materials: (1) Brewer anaerobic culture dish; (2) regular petri dish with bottom either 15 mm. or 10 mm. deep; (3) infusion agar suitable for anaerobes which contains suitable reducing agents, such as the following: 0.2% sodium thioglycollate, 0.1% sodium formaldehyde sufoxylate, and 0.0002% methylene blue.

Method: Pour sterilized medium in bottom of regular petri dish (25 ml. minimum in 10 mm. dish, and 40 ml. minimum in 15 mm. dish). Streak center area from sample or culture. Replace the lid of the regular dish with the Brewer anaerobic lid. (The lid at its periphery, should touch the agar at all points in order that a perfect seal be obtained. In the successfully prepared dish, the agar in the center of the dish remains colorless while the blue color returns to the agar at the edge of the dish due to oxygenation of the

[12]*Brewer anaerobic dish.* Baltimore Biological Laboratory, Baltimore, Md., and Kimble Glass Company, Vineland, New Jersey.

dye which serves as an oxidation reduction potential indicator.) Place plates in the incubator immediately after they are prepared and examine as needed during the incubation period. When transfers are to be made from the plate, break the seal by a slight turn of the lid.

Advantages: A useful, quick method of single plate culture. An extremely simple method which is easy to learn and use. The only trick in the technic is to have sufficient agar in the original dish that a perfect seal is formed when the special lid is added. Recommended for routine use in hospital laboratories, and particularly for mobile laboratories, where anaerobic cultures for pathogens may be encountered. *Disadvantages:* Surface moisture may result in film formation in some instances; this may be reduced by using a porcelain top (see footnote 4) on the regular dish prior to the Brewer anaerobic lid or drying the plates in incubator before streaking. Some organisms apparently are inhibited by the reducing agents. This is not serious since the reports indicate that all pathogenic types are easily cultured by this method. The Brewer anaerobic lids are, at the present time, relatively expensive.

There are other anaerobic systems which are satisfactory as, for example, the Novy jar which depends upon evacuation and gas replacement in a specially designed desiccator. These will not be discussed, however, as they are less commonly used at the present time, and it is believed that the methods discussed above will be satisfactory in most instances.

TECHNICS FOR STUDY OF ANAEROBIC BACTERIA[13]

In the above section the various pieces of apparatus and methods for their use with anaerobic bacteria have been considered. Formulae for the particular media which are recommended may be found in the 9th edition of Leaflet II[14]. The remainder of this Leaflet will be devoted to a discussion of the details of certain technics which should aid the worker who has not had previous experience with anaerobes.

It may not be amiss to insert here a precautionary note concerning the necessity of very careful inspection of the purity of cultures. There are instances on record, in the older literature, where two species grew symbiotically on plate culture with such constancy that recorded observations were made of the colony type of mixture, the investigator being unaware of the existence of more than one type. In all studies concerning obligate anaerobes, a check on the purity of the culture should be made with regard to aerobic contami-

[13]In this Leaflet reference will be made to the "pathogenic group" and the "butyric-butyl group". The former term is used to designate such organisms as *Clostridium tetani, C. septicum, C. histolyticum, C. chauvoei, C. perfringens, C. parabotulinum, C. botulinum* and *C. sporogenes.* In the butyric-butyl group are included *C. butyricum, C. beijerinckii, C. butylicum, C. pasteurianum, C. acetobutylicum, C. felsineum, C. roseum,* and *C. thermosaccharolyticum.*

[14]To be published about February, 1944.

nants. The following test is suggested: For most cultures, streak a glucose nutrient agar slope and incubate it at 37° C.; but for anaerobic species having a lower or higher optimum temperature, incubate a second agar slope at the temperature which is optimum for the anaerobe. If the culture appears free of aerobic types, investigate the purity with respect to anaerobic contaminants. Make repeated platings and scrutinize intensely the colonies which develop.

Preliminary Microscopic Examination

If the sample is suitable, one should make preliminary examination using the Gram stain. The conventional method of staining a smear, heat fixed on a glass slide, should be used, except that the decolorizer should be either 95% ethyl alcohol (*preferred*) or 25 parts acetone and 75 parts ethyl alcohol. The use of greater amounts of acetone must be avoided because of the ease with which anaerobes are decolorized. The usefulness of the Gram method is limited in smears prepared from blood, fibrin or albumin. In samples of pathologic material, large, Gram-positive rods are likely to prove to be anaerobic bacilli, but a final diagnosis must not be based on microscopic observations unsupported by cultural tests. Of the strictly aerobic Gram-positive species, *Bacillus anthracis* Koch is the only usual pathogen. The characteristic morphology of *Clostridium perfringens* (syn. *C. welchii*) and the regularity of its appearance in certain clinical conditions frequently combine to give presumptive evidence of value; similarly, the typical microscopic picture presented by a spore-bearing *C. tetani* culture should be remembered when such forms are encountered in pathologic material. All anaerobic species are non-acid fast; therefore, this stain has no diagnostic importance.

Microscopic Examination of Pure Cultures

GRAM STAIN

If the organism in question will grow within this period, apply the Gram stain to a 16–18 hour culture and observe the same caution with reference to the decolorizer as noted above. Ordinarily the stain is satisfactory when prepared from any enrichment medium in which the organism will grow. In recording the Gram reaction of a new species, state the medium from which the smear was made and the age of the culture.

EXAMINATION FOR MOTILITY

The majority of the spore-forming anaerobic bacilli are motile; the most important exception is *C. perfringens* (*C. welchii*). The technic by which the motility examination is made is often of utmost importance in securing the correct results. *Unless the culture is known to be nonpathogenic, discard all coverslips and slides into a disinfectant solution or sterilize by steam before washing.* Use young cultures (12–18 hours) except as noted. Accept the results of hanging drop or wet-mount preparations under coverslips only if observa-

tion reveals positive motility. If motility is doubtful or appears to be negative, *initiate other procedures.* For example, use a flattened capillary tube sealed at each end. Heat glass tubing, of small diameter, and flatten a small area. Prepare a capillary tube from the flattened section. Draw a small amount of culture into this tube and seal the tube in the flame on both sides of the drop of culture. Examine this preparation with the high power objective. If the motility is still recorded as negative, make further observations on younger (4–6 hour) cultures. For these, examine the 3rd or 4th tube of a serial passage series, using the medium which appears to give the best growth of the culture. Because of the relatively small number of species which are non-motile, considerable caution should be exercised in reporting cultures which appear to be non-motile. Naturally occurring non-motile variants of motile species, however, have been encountered.

FLAGELLA STAIN

For material for preparation of flagella stains use young cultures growing in the medium which is most favorable to the organism being studied. If difficulty is encountered in securing positive slides from cultures known or thought to be motile, consult the directions given by O'Toole (1942) for suggestions in technic which refer particularly to anaerobic bacteria.

CAPSULE STAIN

For the capsule stain one may use any of the conventional methods. The most important capsulated species is *Clostridium perfringens* (*C. welchii*). Material taken from artificially infected laboratory animals generally serves as the origin of smear preparations. If stains from *in vitro* cultures are desired, the medium of Svec and McCoy (See Leaflet II) is useful if other media prove unsuccessful.

DEMONSTRATION OF SPORES

Cultures surviving 20 minutes heating at 80° C. may be presumed to be spore-formers. It is, however, useful to demonstrate the spores microscopically. The exact method of making the spore-stain is of little importance, in comparison with other factors, as each of the common methods (Dorner, Moeller, and malachite green) appears satisfactory. One must, however, pay some attention to the medium in which one expects to induce sporulation. Media containing fermentable carbohydrates are not satisfactory, in general, for the pathogenic group. The media naturally containing carbohydrate (*e.g.*, corn mash or potato infusion), on the other hand, appear ideal for most of the butyric-butyl group. For the pathogens one should use the deep brain, or beef heart, or alkaline egg medium. In some instances spores may be demonstrated within 24–28 hours after inoculation, but, if the culture is negative at this time, older cultures should be examined. Protection from evaporation must be given cultures which are to be incubated longer than

one week. *C. perfringens* (*C. welchii*) appears to be one of the most difficult species in which to demonstrate spores microscopically with regularity. If success is not attained using the above-mentioned media in cultures having the characteristics of this organism, one may use the medium recommended by Svec and McCoy (See Leaflet II).

Since some taxonomic systems give considerable attention to the size and position of the spore, these characteristics should be recorded when the original laboratory examination is made. The characteristic appearance of *Clostridium tetani* spores has been noted above; these are round in shape and borne at the end of a slender vegetative rod. This is almost the only instance in which the picture of the spore and sporangium assumes importance in species diagnosis, and this observation must be supported by cultural or pathologic information as nontoxic organisms of similar microscopic characters occur.

GRANULOSE REACTION

The cells of certain species, particularly during the early stages of spore formation, store granulose. To test for this, add a drop of Lugol's iodine to a wet mount preparation. Cells containing granulose will stain blue or violet while others will appear yellow.

Cultivation Technics[15]

PRELIMINARY ENRICHMENT METHODS

Ordinarily the best method to be followed in initiating growth of an anaerobe from a sample is to inoculate one of the tubed media rather than to proceed directly to plate culture. Certainly this should be done if there is question concerning the possible success of the preliminary culture, and it is advised that parallel tube cultures be inoculated to serve as reserve cultures at the same time the plating is done, if the plating technic is favored. The medium to be used will be a matter of choice, as discussed in Leaflet II (9th Edition), depending upon the nature of the sample. If aerobic contamination is suspected and the anaerobe is thought to be in the spore state, a duplicate primary culture should be heated briefly (boil for one or two minutes, or hold at 80° C. for 20 minutes). This should be a duplicate culture, however, in case the anaerobic form is a non-spore-former or is a spore-former in the vegetative state. Almost all types of tubed media should have the dissolved oxygen driven off by boiling or heating in flowing steam.

For the gas gangrene and tetanus group in infected wounds, Reed and Orr (1941) recommend a technic to those who work in clinical laboratories and examine such material. The technic would appear to involve more cultures than is necessary but the importance of the success of the preliminary culture, and the speed with which it is attained, necessitate the routine suggested. Colonies which appear in the plates are transferred to tubes of thioglycollate medium and species identification begun *immediately. It should be remembered that gas gangrene frequently is a polymicrobic*

[15]The use of vaseline, mineral oil or other materials as a seal at the surface of liquid media is not recommended.

infection and therefore more than one colony type from a single sample is not to be unexpected. With slight modifications their suggestions are as follows:

(1) Inoculate heavily tubes of beef heart medium. Use these subsequently only if the primary plating fails.

(2) Introduce swabs or fragments of tissue into 8 ml. amounts of thioglycollate broth, mix well, and make 1:10, 1:100, and 1:1000 dilutions in the same medium (not saline).

(3) From each dilution prepare surface plates on clear peptone-thioglycollate agar and pour plates in semisolid agar. As an additional or alternate medium, use blood agar; in which case hemolysis, if present, is an additional helpful characteristic. Incubate the plates at 37° C. in a Brewer or McIntosh and Fildes jar. Place a petri dish lid containing granular $CaCl_2$ at the bottom of the stack of plates, and another at the top, to absorb the moisture which forms in the jars. Use the Brewer or Spray plate if an anaerobic jar is not available.

PRELIMINARY PURIFICATION PROCEDURES

It is often difficult to isolate anaerobic bacteria from enrichments which also contain aerobic bacteria. It would be presumed that aerobic bacteria could ordinarily be eliminated merely by the anaerobic environment when this is introduced. Often in practice this is not the case, and other procedures must be instituted. It is of value frequently to attempt partial or complete elimination of the contaminants in tube culture using a liquid medium before plating is done. Materials derived from human or animal sources, other than feces, are usually contaminated with non-sporulating aerobic rods and cocci. Cultures derived from milk, soil, water, grains, feces, etc., contain, in addition, spore-forming aerobes. In fecal and perhaps other samples the contamination may include non-spore-forming anaerobes. If the non-spore-forming anaerobe is *wanted*, then anaerobic plating, and picking of isolated colonies, should be combined with optimum temperature and selective medium to secure the culture. In all cases the original enrichment tube should be preserved in the refrigerator, after growth is evident, until the purification routine is successfully completed. This will insure a supply of starting material should something go wrong with the purification.

Generally one of the easiest practices to be followed to get rid of non-spore-forming types is as follows: Heat subcultures from the contaminated enrichment, retaining the original tube, of course, unheated. Heat the newly inoculated tubes 20 minutes at 80° C. or a shorter time at higher temperatures. Take care to insure the presence of the spores of the anaerobe. Use old cultures in a sugar-free medium as the best source of material to be heated, although other cultures may be satisfactory in special situations.

For enrichments contaminated with spore-forming aerobes the above procedure may not be satisfactory, due to the heat resistance of the aerobic spores. In this case, one may employ dyes as bacteriostatic agents. Nearly all, if not all, aerobic spore-formers are inhibited by crystal violet, and most of the anaerobic types are relatively resistant. Two or three serial transfers may, therefore,

be made in a medium containing this dye (approximately 1–100,000 final concentration) to eliminate the aerobe. The exact concentration of the dye to be used may vary with the medium and the conditions at hand. If used in some of the complex media the effectiveness of the dye may be reduced during sterilization; therefore, the dye should be added to such media after sterilization. Either liquid or solid media may be used.

Another method for elimination of aerobic spore-formers utilizes the fact that while growth of the aerobe may take place in an anaerobic environment the conditions for sporulation are unfavorable. Under such conditions the anaerobe will be expected to sporulate freely. Thus liquid cultures in tubes or plate cultures taken from an anaerobic jar are chosen for material for heating as in the case of the non-spore-forming contaminants.

ISOLATION PROCEDURES

From a purely theoretical viewpoint, microscopic single cell methods of isolation are ideal, but the low percentage of successes with these procedures excludes them from any uses except research. Several reports are in the literature indicating success with anaerobes using the Chambers micromanipulator, or similar instruments, and wherever there is great need for strains of single cell origin, the technic should be attempted. Due to the sensitivity of the vegetative cells toward oxygen, it is recommended that spores be picked rather than vegetative cells. One should use freshly exhausted media showing highly reducing activity for the subcultures and naturally the medium should be suited to the organism being purified. If growth is not evident within the first 48 hours, the tubes may be protected from evaporation and incubated indefinitely. Reputable workers have reported dormancy of spores for six months or longer duration.

In routine problems either plating or deep agar tube methods are available for purification of cultures from the original enrichment tubes. As stated above, the usual procedure in the isolation of anaerobes from samples in which contamination is excessive is best done by attempting partial purification in tube culture. This, however, need not be the case if the population of the sample is dominated by one species. In these the plating routine may be started without the preliminary enrichment procedure. Perhaps a few words should be included concerning details of technic. Since some of the anaerobes tend to spread rapidly over the surface of the agar, in many instances it will be found that "poured" agar plates are to be preferred to plates inoculated by streaking the surface. Two common methods are available for preparing these: (1) melt tubes of the plating medium, cool, and inoculate before pouring; (2) place a small amount of sterile tap water in the culture dish, inoculate, and pour the agar into the dish immediately. If conditions warrant, use crystal violet in the agar. Place the plates in the anaerobic environment as soon as possible. (The size of inoculum to be used will vary so that some practice may be necessary to give

a dilution sufficient that well isolated colonies will appear.) If difficulty is encountered in obtaining discrete colonies, reduce the agar concentration in the plating medium to 0.75 to 1.0%.

Another method is available for colony isolation which may be preferred, particularly if the special apparatus needed for some of the plating methods is not at hand. This method involves the inoculation of a column of medium as mentioned in the opening pages of this Leaflet in the discussion of methods useful to determine whether or not a particular strain is an obligate anaerobe. For isolation purposes the fewer the number of colonies appearing in the medium the better. The percentage of fermentable sugar should be reduced to the lowest amount which gives good growth of the organism in order to prevent the production of gas which may crack the medium. Assuming that we have available a deep tube of agar in which there appear several isolated colonies, two methods of isolation are available: (1) If soft glass tubes are used, cut the glass and break the tube at a short distance below the desired colony. Deposit the agar quickly in a sterile petri dish. Using a hot needle or small blade cut across the plug of agar near the colony and transfer it to a suitable liquid medium. (This method is preferred if the tube shows aerobic contamination in the upper layers.) (2) If Pyrex tubes are used, eject the plug of agar into the sterile dish by applying a Bunsen flame to the bottom end. Before this heat the sides of the tube and sterilize the mouth of the tube in the flame. During the ejection step of the technic, hold the mouth of the tube so that it points directly into the sterile dish. After the column of agar is deposited in the dish, proceed as discussed above.

INOCULATION TECHNICS

The following points of culture transfer and other routine technics are sufficiently different from the procedures used with aerobes so that some note is needed:

Steam or boil most liquid media for a few minutes immediately prior to inoculation in order to drive off oxygen which may have been absorbed following sterilization. Attempt to deliver the inoculum to the *bottom* of the new tube of medium, for it is this portion of the medium which will stay reduced the longest. Although it is possible to initiate growth from a small number of cells, in routine studies use a more adequate inoculum. To facilitate the placing of the inoculum in the bottom of the tube with liquid and semisolid media substitute a Wright or Pasteur pipette (used with small rubber bulbs) for the inoculation needle. By this means transfer a small drop (0.1 or 0.2 ml.) of the culture to the new tube. Use pipette also in the isolation of subsurface colonies particularly from media in which the concentration of agar is reduced. Prepare these pipettes from 6 to 8 inch lengths of sterile 8–9 mm. soft glass tubing (with cotton plug in each end) by applying heat to the center of the glass and pulling to form two capillary pipettes.

In general use a culture from 16–20 hours old. With the pathogenic types this time may be extended a few hours with no harm.

With the butyl-butyric types, however, which sporulate readily in many media, there is a critical period in which the culture is not very satisfactory for transfer purposes. As the culture goes into the spore stage it is less and less suitable until sufficient time elapses for the spores to mature. When spores are present in the inoculum, with these cultures and perhaps others as well, the new tube should be given a heat treatment (80° C. for 20 minutes) *after* inoculation.

Generally, if an anaerobic spore-forming culture is desired in an experiment, inoculate a tube of a favorable medium from a stock culture which contains spores, heat-shock it, and use the resulting culture for the experiment rather than the inoculation of the latter tube or flask directly from the spore containing culture. Maintain the stock culture in the spore state and follow the above transfer routine, rather than carry the anaerobe in a serial passage, and use such cultures for sources of inoculum for experimental flasks or tubes. This is particularly true with the actively fermentative types, where serial passage may yield a culture of undesirable characters—even though it is descended in pure state from a culture that was satisfactory.

Other Methods of Value

STOCK CULTURE METHODS

The anaerobes are susceptible to freezing-drying technic as a means of preservation of cultures over a long period of time as shown by Roe (1940). This technic is unnecessary, however, as species of *Clostridium* are usually viable in spore state over a long period of time. For the pathogenic group, one should use beef heart infusion, alkaline egg medium, and brain mash, with the latter perhaps being the best. With the butyric-butyl group, use plain corn mash or potato infusion. Prepare the plain corn mash in a manner similar to the method given for corn-liver medium with the exception that the liver powder is omitted. Brain medium may be suitable also. (See also Leaflet II, 9th Edition.)

In any medium after all gassing has subsided and spores have been demonstrated microscopically, the tube should be sealed in the flame or the stopper covered to protect the medium from evaporation, and the tube placed in a cool room or refrigerator. Viable subcultures may be obtained from such tubes for months or even years in some instances. Another method which has been used with success is worthy of mention. This involves the storage of cultures on sterile soil: Dry fresh garden soil and sift through a fine mesh screen; add 5% of $CaCO_3$ to neutralize any acidity of the culture. Place soil in tubes in 2 inch columns and autoclave overnight. Test each tube for sterility using both aerobic and anaerobic media. If sterile, add 2 or 3 ml. of a well sporulated culture with a sterile pipette and dry the tube (preferably in a vacuum desiccator). To obtain an active culture from this stock (which may be stored at room temperature) transfer a small amount of the soil to an enrichment medium and heat shock. By the soil stock method a relatively permanent source is available from which cultures may be revived as needed without destroying the stock culture.

SEROLOGICAL REACTIONS

The serological relationships of the spore-forming anaerobes have been reviewed (McCoy and McClung, 1938) and it is suggested that this paper should be consulted as a background and for further references by those who are interested in this topic. The toxin-antitoxin reaction is of value as a taxonomic aid with certain species. In such an instance one takes advantage of the fact that relationships may be established by the success or failure of the reaction of antitoxin, prepared against the toxin of a known organism, with the toxin from the unidentified strain. In some instances the anaerobic species are monotypic with respect to toxin formation. In other species this is not true and subgroups have been established within these species or species groups on the basis of non-cross neutralization tests.

The problem of toxin production may be briefly mentioned. Although studies have been initiated on the possibilities of synthetic media for this purpose, such studies are designed to provide toxin for chemical purification investigations and for production of toxoid. If it is desired to test for the possibility of production of toxin by a particular culture, it is unnecessary to use a synthetic medium since one of the complex media will serve as well and because less difficulty with regard to growth is encountered. For organisms producing the tetanus or botulinus toxin use the beef heart infusion. For the gangrene group use the same medium or glucose meat infusion or the medium of Reed, Orr and Baker (1939). For formulae consult Leaflet II. Use the Berkefeld or Mandler filter to remove cells from the liquid of a 24–72 hour culture. Discard the first 25 ml. of filtrate before collecting the test sample.

For the agglutination reaction, cells for antigen suspensions may be prepared by centrifuging from broth cultures in which maximum growth is attained quickly. For the pathogenic group glucose meat infusion broth or perhaps thioglycollate broth should be used. For the butyric-butyl group, one should employ 1% tryptone broth or yeast infusion broth with 0.5 to 1.0% glucose, with a heavy inoculation from a liver broth culture into deep tubes or bottles of the medium chosen. Care should be taken to collect the cells before excessive slime formation is evident in order to produce a stable antigen.

REFERENCES

Barker, H. A. 1936. Studies upon the methane-producing bacteria. Arch. Mikrob., **7**, 420–438.

Bergey, D. H., Breed, R. S., Murray, E. G. D., and Hitchens, A. P. 1939. Bergey's Manual of Determinative Bacteriology, *5th Ed.* Williams and Wilkins, Baltimore. 1032 pp.

Brewer, J. H. 1942. A new petri dish cover and technique for use in the cultivation of anaerobes and microaerophiles. Science, **95**, 587.

Brown, J. H., and Brewer, J. H. 1938. A method for utilizing illuminating gas in the Brown, Fildes, and McIntosh or other anaerobe jars of the Laidlaw principle. J. Lab. and Clin. Med., **23**, 870–874.

Committee Upon Anaerobic Bacteria and Infections. 1919. Report on the anaerobic infection of wounds and the bacteriological and serological problems arising therefrom. (Gt. Brit.) Med. Research Council, Spec. Rpt. Ser., **39**, 1–182.

Dack, G. M. 1940. Non-spore-forming anaerobic bacteria of medical importance. Bact. Rev., **4**, 227–259.

Fildes, P. 1931. Anaerobic cultivation. Chap. VI *in* System of Bacteriology, Vol. 9, (Gt. Brit.) Med. Research Council.

Fildes, P., and McIntosh, J. 1921. An improved form of McIntosh and Fildes anaerobic jar. Brit. J. Exp. Path., **2**, 153–154.

Griffin, A. M. 1932. A modification of the Buchner method of cultivating anaerobic bacteria. Science, **75**, 416–417.

Hall, I. C. 1922. Differentiation and identification of the sporulating anaerobes. J. Inf. Dis., **30**, 445–504.

Hall, I. C. 1928. Anaerobiosis. Chapter XIII *in* The Newer Knowledge of Bacteriology and Immunology. *Edited by* Jordon, E. O., and Falk, I. S. Univ. of Chicago Press, Chicago.

Hall, I. C. 1929. A review of the development and application of physical and chemical principles in the cultivation of obligately anaerobic bacteria. J. Bact., **17**, 255–301.

Heller, H. H. 1921. Principles concerning the isolation of anaerobes. Studies in pathogenic anaerobes. II. J. Bact., **6**, 445–470.

Hewitt, L. F. 1937. Oxidation-reduction potentials in bacteriology and biochemistry, *4th Ed.* London County Council.

Hibler, E. von. 1908. Untersuchungen über die pathogenen Anäeroben, über die anatomischer und histologischen Veränderung bei den durch sie bedingten Infektionskrankungen des Menschen sowie der Tiere und über einige nichtpathogene Anäerobenarten. Gustav Fischer, Jena. 438 pp.

Knight, B. C. J. G. 1931. Oxidation-reduction potential measurement in cultures and culture media. Chapter XIII *in* System of Bacteriology, Vol. **9**, (Gt. Brit.) Med. Research Council.

Knorr, M. 1923. Ergebnisse neurer Arbeiten über krankheitserregende Anaerobien. I. Teil. Krankheitserregende anaerobe Sporenbildner, ausschliesslich Tetanus und Botulinus. Zentbl. Gesam. Hyg., **4**, 81–100, 161–180.

Knorr, M. 1924. Ergebnisse neuerer Arbeiten über krankheitserregende Anaerobien. II. Teil, 1: Botulismus. Zentbl. Gesam. Hyg., **7**, 161–171, 241–253.

Laidlaw, P. P. 1915. Some simple anaerobic methods. Brit. Med. J., **1**, 497–498.

McClung, L. S., and McCoy, E. 1941. The anaerobic bacteria and their activities in nature and disease: a subject bibliography. Suppl. 1: Literature for 1938 and 1939. Univ. of California Press. *xxii* and 244 pp.

McClung, L. S., McCoy, E., and Fred, E. B. 1935. Studies on anaerobic bacteria. II. Further extensive uses of the vegetable tissue anaerobic system. Zentbl. Bakt., II Abt., **91**, 225–227.

McCoy, E., Fred, E. B., Peterson, W. H., and Hastings, E. G. 1926. A cultural study of the acetone butyl alcohol organism. J. Inf. Dis., **39**, 457–483.

McCoy, E., Fred, E. B., Peterson, W. H., and Hastings, E. G. 1930. A cultural study of certain anaerobic butyric acid-forming bacteria. J. Inf. Dis., **46**, 118–137.

McCoy, E., and McClung, L. S. 1938. Serological relations among the spore-forming anaerobic bacteria. Bact. Rev., **2**, 47–97.

McCoy, E., and McClung, L. S. 1939. The anaerobic bacteria and their activities in nature and disease: a subject bibliography (*in two volumes*). Univ. of California Press. *xxiii* and 295 pp.; *xi* and 602 pp.

McIntosh, J. 1917. The classification and study of the anaerobic bacteria of war wounds. (Gt. Brit.) Med. Research Council, Spec. Rpt. Ser., **12**, 1–58.

Marshall, M. S., and Nordby, H. 1942. Anaerobic plates. J. Bact., **44**, 619.

Meyer, K. F. 1928. Botulismus. *In* Kolle, W., Krause, R., und Uhlenhuth, P. Handbuch der pathogenen Mikroorganismen, *3 Aufl.*, **4**, 1269–2364.

Mueller, J. H., and Miller, P. A. 1941. A modification of Rosenthal's chromium-sulfuric acid method for anaerobic cultures. J. Bact., **41**, 301–303.

O'Toole, E. 1942. Flagella staining of anaerobic bacilli. Stain Techn., **17**, 33–40.

Prévot, A.-R. 1924. Les streptocoques anaérobies. Thesis, Paris. 144 pp.

Prévot, A.-R. 1925. Les streptocoques anaérobies. Ann. Inst. Pasteur, **39**, 417–447.

Prévot, A.-R. 1938. Études de systématique bactérienne. III. Invalidité du genre *Bacteroides* Castellani et Chalmers. Démembrement et reclassification. Ann. Inst. Pasteur, **60**, 285–307.

Prévot, A.-R. 1940a. Études de systématique bactérienne. V. Essai de classification des vibrions anaérobies. Ann. Inst. Pasteur, **64**, 117–125.

Prévot, A.-R. 1940b. Manual de Classification et de Détermination des Bacteries Anaérobies. Masson et Cie., Paris. 223 pp.

Reed, G. B., and Orr, J. H. 1941. Rapid identification of gas gangrene anaerobes. War Med., **1**, 493–510.

Reed, G. B., and Orr, J. H. 1943. Cultivation of anaerobes and oxidation-reduction potentials. J. Bact., **45**, 309–320.

Reed, G. B., Orr, J. H., and Baker, M. C. 1939. Gas-gangrene-toxin production Soc. Expt. Biol. and Med., Proc., **42**, 620–621.

Robertson, M., and O'Brien, R. A. 1929. The organisms associated with gas gangrene. Chap. IX *in* System of Bacteriology, Vol. **3**, (Gt. Brit.) Med. Research Council.

Roe, A. F. 1940. Report on viability of 200 cultures of anaerobes desiccated for six years. J. Bact., **39**, 11–12.

Rosenthal, L. 1937. "Chromium-sulfuric acid" method for anaerobic cultures J. Bact., **34**, 317–320.

Snieszko, S. 1930. The growth of anaerobic bacteria in petri dish cultures. Centbl. Bakt., II Abt., **82**, 109–110.

Soule, M. H. 1932. Anaerobic technic. J. Lab. and Clin. Med., **17**, 519–529.

Spray, R. S. 1930. An improved anaerobic culture dish. J. Lab. and Clin. Med., **16**, 203–206.

Spray, R. S. 1936. Semisolid media for cultivation and identification of the sporulating anaerobes. J. Bact., **32**, 135–155.

Weinberg, M., and Ginsbourg, B. 1927. Données Récentes sur les Microbes Anaérobies et leur Rôle en Pathologie. Masson et Cie., Paris. 291 pp.

Weinberg, M., and Séguin, P. 1918. La Gangrène Gazeuse. Masson et Cie., Paris.

Weinberg, M., Nativelle, R., and Prévot, A.-R. 1937. Les Microbes Anaérobies Masson et Cie., Paris. 1186 pp.

Zeissler, J. 1930. Anaërobenzuchtung. *In* Kolle, W., Krause, R., und Uhlenhuth, P., Handbuch der pathogenen Mikroorganismen, *3 Aufl.*, **10**, 35–144.

Zeissler, J., und Rassfeld, L. 1928. Die anaerobe Sporenflora der europaischen Kriegsschauplätze 1917. Veröffentl. aus der Kriegs- und Konstitutionspathologie, **5**, Heft 2. 99 pp.

LEAFLET IV

STAINING METHODS

9th EDITION

Pure Culture Study of Bacteria. Vol. 14, No. 2-3

AUGUST 1946

Committee members assisting in the revision: Barnett Cohen, M. W. Jennison, L. S. McClung, and A. J. Riker

LEAFLET IV

STAINING METHODS

9th Edition

General Principles

The staining of bacteria depends in general upon the same properties of dyes as does the staining of animal or plant tissue for histological purposes. Short discussions of the nature of dyes, with special reference to staining are given elsewhere (Conn, 1940; Churchman, 1928) and only the briefest summary of the subject need be given here.

All bacterial dyes are synthetic products—anilin dyes, or coal-tar dyes, as they are generally called. Although the synthetic dyes vary greatly in their chemical nature and staining properties, they are for practical purposes often divided into two general groups, the acid dyes and the basic dyes. These terms do not mean that the dyes in question are free acids or free bases. The free color acids and bases, when obtainable, are colored, to be sure, but they are often insoluble in water, and rarely have appreciable staining action—i. e., the colors do not "stick." The salts of these compounds, on the other hand, are more soluble, penetrate better, and stain more permanently; they are the true dyes.

An acid dye is the salt of a color acid, a basic dye the salt of a color base. In other words, acid dyes owe their colored properties to the anion, basic dyes to the cation. The actual reaction of an aqueous solution of a dye, however, depends on several factors; and an acid dye may well be basic in reaction, while a basic dye may be acid. This is because the reaction of such a solution depends on the relative strengths of the dye ion and of the anion or cation with which it is combined in the dye salt.

Basic dyes have greatest affinity for the nuclei of cells, probably because of the acid nature of the nuclear material. Acid dyes have a stronger tendency to combine with the cytoplasm. As bacteria do not show typical cell structure and the nuclear material seems to be distributed throughout their bodies, they tend to stain fairly uniformly with nuclear, i. e., the basic, dyes. Hence, the stains in common use by the bacteriologists are rarely acid dyes.

PREPARATION OF SMEARS

Pure cultures of bacteria can ordinarily be prepared for staining by the simple process of making an aqueous suspension and drying a drop of it on a slide or cover glass, without any fixation other than gentle heat. The use of this simple procedure depends upon the fact that most bacteria, because of their small size or their stiff walls, can be dried without great distortion. For this reason it is not

usually necessary, as with higher organisms, to coagulate the tissues before microscopic preparations can be made; although it has been well demonstrated that for accurate determinations of size and shape of the cells, some form of fixation other than heat is needed.

The best bacterial smears are usually made by removing a small amount of surface growth from some solid medium and mixing it with distilled water. It is often possible to use a drop of a culture growing in a liquid medium, but such a smear is not always so satisfactory, since certain constituents of the medium may prevent the bacteria from adhering to the slide or may interfere with the staining.

The suspension used should always be sufficiently dilute. Ordinarily, only a faint turbidity should be visible to the naked eye; for it is always best to avoid the occurrence on the slides of solid masses of bacteria, piled one on top of the other. If a smear after staining does not show any portions where the bacteria are well separated one from another, a new, more dilute smear should be made. This is particularly important in the case of the Gram stain, or flagella staining.

The usual method of fixing the suspension to the slide or cover glass is to pass it rapidly after drying through a Bunsen flame two or three times. Another very satisfactory method is to allow the drop of material to dry on a slide lying on a flat, moderately hot surface, such as a plate of some non-rusting metal resting on a boiling water bath. With many bacteria an aqueous suspension of the surface growth from agar can be dried in the air at room temperature and stained without any fixing; this method is not universally successful, however.

For special staining procedures special methods of making bacterial preparations are necessary, sometimes calling for fixing solutions rather than heat. It is beyond the scope of this leaflet, however, to discuss them here, but it must be recognized that the technic described above for staining dried smears is too crude for accurate measurements of cells or for studying their cytological details.

It is also beyond the scope of this publication to give staining methods for other than pure culture work, although a few (e.g., blood stains) have been given in previous editions.*

In using any of the methods it must be remembered that blind adherence to a staining technic is no guarantee that the result will be satisfactory. Even experienced workers sometimes discover to their dismay that they took too much for granted as to the purity of their reagents, cleanliness of slides and covers, or proper compounding of the staining solutions. A technic should, therefore, be checked upon known organisms as controls. It is, furthermore, important to know that the solutions and water used for dilution are reasonably free from bacteria and their spores.

*Those interested in other stains for microorganisms and for blood are referred to the following leaflets of Staining Procedures (Conn and Darrow, 1943–5):

I D. Miscellaneous methods (blood, bone, marrow, fat).

III A. Stains for microorganisms in smears.

III B. Stains for microorganisms in sections.

These leaflets can be purchased separately and are punched so as to fit the cover to this Manual.

STAINING FORMULAE

There has always been a surprising amount of inaccuracy in the literature concerning staining solutions. This is due to a variety of causes: indefiniteness in the original publication; mistakes of copying by later authors; modifications of the original which are not described as modifications and come later to be ascribed to the original author; failure of authors to cite references when giving their methods. For such reasons it has proved necessary in this publication to give in many instances both the original (rather indefinite) formula and an emended formula as interpreted by the Committee. The Committee, however, *assumes no responsibility for the identity of the two, and offers the emendation merely to prevent the perpetuation of formulae which are clearly ambiguous or indefinite as to their ingredients.* Recent cooperation between this Committee, the Biological Stain Commission, and the National Formulary Committee of the American Pharmaceutical Association, has resulted in the virtual adoption of these emended formulae.

In the present edition of this leaflet the practice is still continued of giving both the original and the emended formulae in such instances. It is anticipated, however, that the latter will be regarded as sufficiently standard, in a few years, so that the original formulae can be dropped in future editions.

In early editions of this leaflet staining formulae and methods were merely taken from the literature without any endorsement by the Committee. At present, greater experience in such matters permits the Committee to recommend certain of the procedures, and they are now grouped according to whether or not they are thus endorsed. Several of the less frequently used methods formerly given are now omitted. One or two new methods are included among those recommended by the Committee.

Staining Schedule. *Tap vs. distilled water.* When washing slides after applying any stain, tap water is ordinarily more convenient to use than distilled water; and in the staining schedules that follow, tap water is specified in those instances where its use is considered to be ordinarily unobjectionable. It must be remembered, however, that the *use of distilled water is never contraindicated* for such purposes; and many bacteriologists prefer it for all steps where washing is called for, because it is not subject to variation in composition, buffer content, etc.

General Bacterial Stains—Recommended Procedures

ZIEHL'S CARBOL-FUCHSIN

OLD STATEMENT OF FORMULA		EMENDED STATEMENT OF FORMULA		
		Solution A		
Sat. alc. sol. basic fuchsin	10 ml.	Basic fuchsin (90% dye content)[1]	0.3	g.
5% sol. carbolic acid	100 ml.	Ethyl alcohol (95%)	10	ml
		Solution B		
		Phenol	5	g.
		Distilled water	95	ml.
		Mix Solutions A and B.		

[1]It is not necessary that dry stains of the exact dye content specified be used in this or in the following formulae. Samples of higher or lower dye content may be employed by making the proper adjustment in the quantity used.

AMMONIUM OXALATE CRYSTAL VIOLET (HUCKER'S)

Solution A		Solution B	
Crystal violet (90% dye content)	2 g.	Ammonium oxalate	0.8 g.
Ethyl alcohol (95%)	20 ml.	Distilled water	80 ml.

Mix solutions A and B.

CRYSTAL VIOLET IN DILUTE ALCOHOL

Crystal violet (90% dye content)	2 g.
Ethyl alcohol (95%)	20 ml.
Distilled water	80 ml.

LOEFFLER'S ALKALINE METHYLENE BLUE

ORIGINAL STATEMENT OF FORMULA		EMENDED STATEMENT	
		Solution A	
Conc. sol. methylene blue in alcohol	30 ml.	Methylene blue (90% dye content)	0.3 g.
Sol. KOH in distilled water (1:10,000)	100 ml.	Ethyl alcohol (95%)	30 ml.
		Solution B	
		Dilute KOH (0.01% by weight)	100 ml.

Mix Solutions A and B.

METHYLENE BLUE IN DILUTE ALCOHOL

Methylene blue (90% dye content)	0.3 g.
Ethyl alcohol (95%)	30 ml.
Distilled water	100 ml.

CARBOL ROSE BENGAL

Rose Bengal (80% dye content)	1 g.
Phenol (5% aqueous solution)	100 ml.
$CaCl_2$	0.01–0.03 g.

(The amount of $CaCl_2$ added determines the intensity of staining.)

Staining schedule: Follow the general procedure given under "Preparation of Smears", p. 3–4 above, allowing 5–60 seconds for application of the stain. Overstaining rarely occurs except with carbol fuchsin; understaining does not have to be feared except with rose Bengal.

Results: The results depend on which of the above staining fluids is selected. They are listed in the order of intensity of action; i.e. carbol fuchsin gives the most intense stain, and is not indicated when selective staining is desired or when much debris is present on the slide. The crystal violet solutions are very good for routine purposes. The methylene blue solutions are much more selective, with special affinity for metachromatic granules. The rose Bengal solution is much less commonly used; it is specially valuable when mucus or colloidal organic material is present, as such material is not ordinarily stained by it.

General Bacterial Stains—Alternate Procedures

Kinyoun's Carbol Fuchsin

Basic fuchsin (dye content not specified; probably 90%)	4 g.
Phenol crystals	8 g.
Ethyl alcohol (95%)	20 ml.
Distilled water	100 ml.

This formula is preferred in some quarters to the Ziehl carbol fuchsin. It is attributed to Kinyoun, but the reference to its original publication has not been located.

Carbol Crystal Violet (Nicolle)

ORIGINAL STATEMENT OF FORMULA	EMENDED STATEMENT
	Solution A
Sat. alc. gentian violet..... 10 ml.	Crystal violet (90% dye content) 0.4 g.
1% aqu. sol. phenol.....100 ml.	Ethyl alcohol (95%)..... 10 ml.
	Solution B
	Phenol..... 1 g.
	Distilled water.....100 ml.
	Mix solutions A and B.

This formula is sometimes preferred either as a general stain or in the Gram technic If properly prepared it is permanent; but it has a tendency to gelatiniz · if the amount of dye is too great. To prevent this sort of deterioration the quantity of dye in the above amended formula has been reduced to 0.4 g. from the 1.0 g. recommended in previous editions of this leaflet. Even when the solution is so prepared as to be permanent, however, it seems to have no advantage over the ammonium oxalate crystal violet given above.

Anilin "Gentian Violet" (Ehrlich)

ORIGINAL STATEMENT OF FORMULA	EMENDED STATEMENT
	Solution A
Sat. alc. sol. gentian violet.....5–20 ml.	Crystal violet (90% dye content) 1.2 g.
Anilin water (2 ml. anilin shaken with 98 ml. water and filtered) 100 ml.	Ethyl alcohol (95%).....12 ml.
	Solution B
	Anilin..... 2 ml.
	Distilled water.....98 ml.
	Shake and allow to stand for a few minutes, then filter.
	Mix Solutions A and B.

This formula is given largely for its historic interest. It is a quite unstable solution, and has no special value today. It was, however, one of the first important bacterial staining fluids and was formerly regarded as the standard formula for the Gram stain. It is not, however, certain what was the "anilin gentian violet" originally employed in the Gram stain, even though ascribed to Ehrlich. As a matter of fact Ehrlich seems to be properly credited only with the idea of using anilin water in the formula, as he apparently did not recommend any one definite formula.

Negative Staining of Bacteria—Recommended Procedures

Dorner's nigrosin solution

Nigrosin, water soluble (nigrosin B Grübler recommended by Dorner; American nigrosins certified by Commission on Standardization of Biological Stains ordinarily satisfactory)..... 10 g.
Distilled water.....100 ml.
Immerse in boiling water bath for 30 minutes; then add as preservative:
Formalin..... 0.5 ml.
Filter twice through double filter paper and store in serological test tubes, about 5 ml. to the tube.

This staining solution is used for the negative demonstration of bacteria, in place of the Burri India ink. For its use in Dorner's spore stain, see p. IV46-11.

Staining schedule:

1. Mix a loopful of the bacterial suspension on the slide with an equal amount of the staining solution. (If prepared from growth on solid media, the suspension must not be too heavy.)
2. Allow the mixture to dry in the air, and examine under microscope.

Results: Unstained cells in a background which is an even dark gray if the preparation is well made.

BENIANS' CONGO RED

Congo red (80% dye content) ..... 2 g.
Distilled water ..... 100 ml.

Staining schedule:

1. Place a drop of the above staining fluid on a slide.
2. Mix culture with the drop and spread out into a rather thick film.
3. After film has dried, wash with 1% HCl.
4. Dry, either in the air or by blotting.

Results: Cells unstained in a blue background. Good results are not to be expected from broth cultures or from cultures in salt solutions unless the cells are first removed by centrifuging.

The Gram Stain—Recommended Procedures

There are numerous modifications of the Gram stain, many of which have been listed by Hucker and Conn (1923, 1927). The two modifications given below have proved especially useful to the Committee. The Hucker modification is valuable for staining smears of pure cultures, that of Kopeloff and Beerman for preparations of body discharges such as gonorrhoeal pus, also for pure cultures of strongly acid-forming organisms. The latter is itself a variation of the modification by Burke (1921).

HUCKER MODIFICATION

AMMONIUM OXALATE CRYSTAL VIOLET
(See p. IV46-6)

GRAM'S MODIFICATION OF LUGOL'S SOLUTION

Iodine ..... 1 g.
KI ..... 2 g.
Distilled water ..... 300 ml.

COUNTERSTAIN

Safranin O (2.5% solution in 95% ethyl alcohol) ..... 10 ml.
Distilled water ..... 100 ml.

Staining schedule:

1. Stain smears 1 min. with ammonium oxalate crystal violet. This formula has sometimes been found to give too intense staining, so that certain Gram-negative organisms (e.g. the gonococcus) do not properly decolorize. If this trouble is encountered, it may be avoided by using less crystal violet.
2. Wash in tap water.
3. Immerse 1 min. in iodine solution.
4. Wash in tap water and blot dry.
5. Decolorize 30 sec. with gentle agitation, in 95% ethyl alcohol. Blot dry.
6. Counterstain 10 sec. in the above safranin solution.
7. Wash in tap water.
8. Dry and examine.

Results: Gram-positive organisms, blue; Gram-negative organisms, red.

BURKE AND KOPELOFF-BEERMAN MODIFICATIONS

ALKALINE GENTIAN VIOLET

Solution A		Solution B	
Gentian or crystal violet[2]	1 g.	$NaHCO_3$	1 g.
Distilled water	100 ml.	Distilled water	20 ml.

BURKE'S IODINE SOLUTION

Iodine, 1 g.; KI, 2 g.; distilled water, 100 ml.

KOPELOFF AND BEERMAN'S IODINE SOLUTION

Iodine	2 g.
Normal NaOH (40.01 g. per liter)	10 ml

After the iodine is dissolved, make up to 100 ml. with distilled water.

BURKE'S COUNTERSTAIN

Safranin O (85% dye content), 2 g.; distilled water, 100 ml.

KOPELOFF AND BEERMAN'S COUNTERSTAIN

Basic fuchsin (90% dye content), 0.1 g.; distilled water, 100 ml.

Staining schedule:

1. Dry thinly spread films in the air without heat.
2. Flood with Solution A; mix on the slide with 2–3 drops (or more, depending on size of flooded area) of Solution B, and allow to stand 2–3 min.

   Kopeloff and Beerman mix the two solutions in advance, 1.5 ml. Sol. A to 0.4 ml. Sol. B, and allow to stay on slide 5 min. or more.
3. Rinse with either of the above iodine solutions. (The Committee indicates no preference between the two; some workers prefer one, some the other.)
4. Cover with fresh iodine solution and let stand 2 min. or longer.
5. Rinse with tap water; then blot water from surface of smear, *without drying.* (Kopeloff and Beerman omit the washing.) The amount of drying is important in this step. One must get rid of all free water, but not allow the cells to dry.
6. Follow the blotting very quickly with decolorization in ether and acetone (1 vol. ether to 1–3 vol. acetone), adding to the slide drop by drop until practically no color comes off in the drippings (usually less than 10 sec.) In this step the speed of decolorization can be varied by varying the ratio of ether to acetone; the more acetone the more rapid the process. It is sometimes desirable to slow down the process by using a ratio of 1:1.
7. Dry in the air.
8. Counterstain 5–10 sec. in one of the above given counterstains. Burke's (i.e. safranin) is preferred. The Kopeloff and Beerman counterstain is too powerful to be used when the shorter staining time recommended by Burke is followed.

[2]The authors specify either crystal violet or methyl violet 6B. Probably any of the gentian violets now sold under the Commission certification are satisfactory; i. e. either crystal violet or one of the bluer grades of methyl violet (e. g., methyl violet 2B).

9. Wash in tap water.
10. Dry and examine.

Results: Gram-positive organisms, blue; Gram-negative organisms, red. This technic is claimed to have the advantage of not giving false positives due to vacuolar bodies that resist decolorization by other Gram-staining procedures.

INTERPRETATION OF THE GRAM STAIN

A word of caution is necessary as to the interpretation of the Gram stain. The test is often regarded with unjustified finality because organisms are generally described as being either Gram-positive or Gram-negative. Many organisms, however, actually are Gram-variable. Hence, one should never give the Gram reaction of an unknown organism on the basis of a single test. He should repeat the procedure on cultures having different ages and should use more than one staining technique in order to determine the constancy of the organism toward the stain. Two phenomena deserve consideration. (1) Henry & Stacey (1943) and Bartholomew and Umbreit (1944) have shown that Gram-positive organisms can be made Gram-negative by treatment with ribonuclease, and that their Gram-positive reaction can be restored subsequently by treatment with magnesium ribonucleate. (2) Some organisms have granules which resist decolorization and which may cause misinterpretation. Such observations show that the Gram stain does not always give a clear cut reaction and that the results must be interpreted with care.

Acid-fast Staining—Recommended Procedure

ZIEHL-NEELSEN METHOD
Ziehl (1882); Neelsen (1883)

Staining schedule:

1. Stain dried smears 3–5 min. with Ziehl's carbol fuchsin (p. 5), applying enough heat for gentle steaming.
2. Rinse in tap water.
3. Decolorize in 95% ethyl alcohol, containing 3% by volume of conc. HCl, until only a suggestion of pink remains.
4. Wash in tap water.
5. Counterstain with one of the methylene blue solutions given on p. 6.
6. Wash in tap water.
7. Dry and examine.

Results: Acid-fast organisms, red; others, blue.

Acid-fast Staining—Alternate Procedures

Fluorescence Method
Richards and Miller (1941)

Although this method is not of special importance in pure culture work, special mention should be made of it because of the amount of attention now given to it in diagnostic work. Its real advantage is that it can be used with relatively low magnification, and the large fields that can be examined assure positive diagnoses in cases where the numbers of tubercle organisms are few.

Solution A		Solution B	
Auramine O (90% dye content)	0.1 g.	Ethyl alcohol (70%)	100 ml.
Liquefied phenol	3 ml.	Conc. HCl	0.5 ml.
Distilled water	97 ml.	NaCl	0.5 g.

Staining schedule:

1. Stain dried smears 2–3 min. in Solution A.
2. Wash in tap water.
3. Destain 3–5 min. in Solution B, freshly prepared.
4. Dry, and examine under a monocular microscope, using 8 mm. dry objective and a 20× ocular; illumination should be a low voltage, high amperage microscope lamp, supplied with a blue (ultraviolet transmitting) filter, a complementary yellow filter having been provided for the ocular.

Results: Acid-fast bacteria, bright yellow, fluorescent; other organisms, not visible; background, nearly black.

Much's Method
Much (1907)

Much's method No. 2, which is now quite widely used, employs carbol gentian violet of essentially the formula given on page IV46-5 for carbol fuchsin except that in the place of basic fuchsin the author calls for methyl violet BN. Preparations are stained cold for 24 hours or by gentle application of heat until steaming. They are then washed in water and treated with Lugol's iodine (see p. IV46-8) from 1 to 5 minutes. After a second washing they are treated with 5% nitric acid for 1 minute followed by 3% hydrochloric acid for 10 seconds. They are then decolorized 1 minute in equal parts of acetone and 95% ethyl alcohol. Weiss (1909) has modified this procedure by staining with a mixture of 3 parts of carbol fuchsin to 1 part of carbol gentian violet and counterstaining with 1% aqueous safranin (5 to 10 seconds) or with Bismarck brown (1 minute). The counterstain is applied immediately after the decolorization, the acetone-alcohol being removed merely by blotting. In some laboratories this method of counterstaining is employed following the Much technic with carbol gentian violet alone for the primary stain.

Cooper's Method
Cooper (1926)

The Cooper method calls for staining in Ziehl's carbol fuchsin to which 3% of a 10% aqueous sodium chloride solution is added just before use. Smears are stained either by steaming 3 to 4 minutes, then allowing them to cool until a precipitate forms, or else by standing overnight in a 37° incubator and cooling in an ice box for 20 minutes to allow precipitation to occur. After the precipitation, the smears are washed with tap water and decolorized 1 to 10 minutes in acid alcohol (5 ml. of nitric acid, sp. gr. 1.42, to 95 ml. of 95% ethyl alcohol); washed again with water, and finally for 1 minute with 95% ethyl alcohol. They are counterstained with 1% brilliant green, or if the smear is heavy, with a greater dilution of this same stain; washed with water, dried, and examined.

Spore Staining—Recommended Procedures

Dorner's method
Dorner (1922, 1926)

Staining schedule:

1. Make a heavy suspension of the organism in 2–3 drops of distilled water in a small test tube.
2. Add an equal quantity of freshly filtered Ziehl's carbol fuchsin (p. 5).
3. Allow the mixture to stand in a boiling water bath 10 min. or more.
4. On a cover slip or slide mix one loopful of the stained preparation with one loopful of Dorner's nigrosin solution (p. 7).
5. Smear as thinly as possible and do not dry too slowly.

Note: If even backgrounds for exhibiting or photographing are required, especially in the case of slime-producing bacteria, the following procedure is recommended:

1. Make the suspension in 0.5 ml. nutrient broth or water.
2. Add 1 ml. of 10% gelatin solution.
3. Add 1 ml. of carbol fuchsin and stain as in (1) and (2) above.
4. Wash out the colloids with warm tap water, with the help of centrifuge or sedimentation.
5. Mix with nigrosin and proceed as above.

Results: Spores, red; vegetative cells, unstained; background, gray.

DORNER METHOD—SNYDER MODIFICATION
Snyder (1934)

Staining schedule:

1. Prepare a dried smear on a slide and cover with a small piece of blotting paper.
2. Saturate blotting paper with freshly filtered Ziehl's carbol fuchsin (p 5).
3. Allow to steam 5–10 min., keeping paper moist by adding more staining fluid.
4. For neat preparations, decolorize instantaneously with 95% ethyl alcohol (but omit this step if the organisms do not hold color well.)
5. Wash with tap water.
6. Apply a drop of saturated aqueous nigrosin (or Dorner's fluid) and spread evenly.
7. Allow slide to dry quickly with gentle heat, without prior washing.

Results: Same as with original method; but this modification proves applicable to some bacteria (e.g. *Bacillus subtilis*) that are difficult to stain by Dorner's technic.

CONKLIN'S MODIFICATION OF WIRTZ METHOD
Wirtz (1908); Conklin (1934)

Staining schedule:

1. Make smears as usual and fix by heat.
2. Flood slide with 5% aqueous malachite green, and steam for 10 minutes, keeping slide flooded by addition of fresh staining fluid.
3. Wash 30 sec. in running water.
4. Counterstain 1 min. with 5% aqueous mercurochrome.
5. Wash in running water.
6. Blot dry and examine.

Results: Spores, green; rest of cell, red. Trouble is sometimes experienced with the green fading after the slides have stood a few days. Apparently this is due to an alkaline reaction and can be prevented by treating the slides in acid before making the smears. (The alkalinity may be due to an invisible film of soap or washing powder.)

Spore Staining—Alternate Procedure

Schaeffer-Fulton Modification of Wirtz Method
Schaeffer & Fulton (1933)

Bacterial smears are made as usual and fixed in a flame. They are flooded with 5% aqueous malachite green for 30 to 60 seconds, and heated to steaming three or four times. The excess stain is washed off in running water for about half a minute, and 0.5% aqueous safranin is added for about 30 seconds. The smears are then washed and blotted. The spores should be stained green, the rest of the cells red.

Staining the Diphtheria Organism—Recommended Procedures

Various special procedures have been devised for staining the diphtheria organism in such a manner as to render it distinctive in appearance by differentiation of its characteristic metachromatic granules.

STAINING WITH METHYLENE BLUE

Staining schedule:

1. Prepare smear as usual, and fix with gentle heat.
2. Stain for a few seconds with either of the methylene blue solutions (i.e. Loeffler's, or dilute alcoholic) given on p. 6.
3. Wash in tap water.
4. Dry and examine.

Results: Metachromatic granules, dark blue to violet; bacteria without such granules, evenly stained. The picture varies a little according to which of the two methylene blue solutions is employed. The Loeffler formula gives purplish shades of staining because of the oxidation of methylene blue caused by the alkali. Some users consider the polychrome effect thus obtained to give better differentiation; others think the metachromatic granules show more sharply with the clear blue of the unpolychromed dye.

ALBERT'S DIPHTHERIA STAIN
Albert (1920)

Toluidine blue	0.15 g.
Methyl green	0.20 g.
Acetic acid (glacial)	1 ml.
Ethyl alcohol (95%)	2 ml.
Distilled water	100 ml.

LAYBOURN'S MODIFICATION

Laybourn (1924) has modified the Albert stain by replacing the methyl green with an equal amount of malachite green.

Staining schedule:

1. Make smears as usual and fix with gentle heat.
2. Stain 5 min. in either Albert's staining fluid or Laybourn's modification of it. The latter is claimed to give deeper staining of both granules and body of the cells, without lessening the contrast between them.
3. Drain without washing.
4. Treat 1 min. in a modified Lugol's solution (iodine, 2 g.; KI, 3 g.; distilled water, 300 ml.).

5. Wash briefly in tap water.
6. Blot with filter paper, and examine.

Results: Metachromatic granules, black; bars of diphtheria cells, dark green to black; body of cells, light green.

LJUBINSKY STAIN
(from Blumenthal and Lipskerow, 1905)

ORIGINAL FORMULA		EMENDED FORMULA	
Solution A		Solution A	
Pyoktanin (Merck)	0.25 g.	Methyl violet 2B or crystal violet (85% dye content)	0.25 g.
5% acetic acid	100 ml.	Glacial acetic acid	5 ml.
		Distilled water	95 ml.
Solution B		Solution B	
0.1% vesuvin		Bismarck brown Y	0.1 g.
		Distilled water	100 ml.

Staining schedule:

1. Make smears as usual and fix with gentle heat.
2. Stain 30 sec. to 2 min. in Solution A.
3. Wash in tap water.
4. Stain 30 sec. with solution B.
5. Wash in tap water.
6. Dry and examine.

Results: Metachromatic granules, dark blue or black; rest of cell, reddish or yellowish.

Staining the Diphtheria Organism—Alternate Procedures

Neisser's Diphtheria Stain
Neisser (1903)

Solution No. 1		Solution No. 2	
Methylene blue (dye content not specified; probably 90%)	1 g.	Crystal violet (dye content not specified; probably 85%)	1 g.
Alcohol (e. g., 95%)	20 ml.	Alcohol (e. g., 95%)	10 ml.
Acetic acid (glacial)	50 ml.	Distilled water	300 ml.
Distilled water	1000 ml.	Solution No. 3	
Mix, and agitate until dye is dissolved.		Chrysoidin	1 or 2 g.
		Hot water	300 ml.
		Filter after dissolving	

Dried films are stained 10 seconds in a mixture of 2 parts of Solution No. 1 and 1 part of Solution No. 2. Wash. Stain 10 seconds in Solution No. 3. Wash briefly in water, or not at all. Blot dry.

Ponder's Diphtheria Stain
Ponder (1912); Kinyoun (1915)

	Original formula	As modified by Kinyoun
Toluidine blue	0.02 g.	0.1 g.
Azure 1	———	0.01 g.
Methylene blue	———	0.01 g.
Glacial acetic acid	1 ml.	1 ml.
Ethyl alcohol (see below)	2 ml.	5 ml.
Distilled water	100 ml.	120 ml.

Dissolve the dyes in the alcohol, add the water, then the acid and let stand 24 hours before using. Do not filter. After prolonged standing, action may be intensified by adding 1 or 2 drops of glacial acetic acid.

According to Kinyoun, smears are fixed with heat, allowed to cool and stained 2–7 minutes.

In the source of the original formula above cited, absolute alcohol is specified; Kinyoun calls for 95% alcohol. On theoretical grounds, indeed, absolute alcohol is not indicated and the 95% strength may well be substituted even in the original formula. Although the Committee has had no personal experience with either formula, information is at hand indicating the superiority of the Kinyoun modification.

Flagella Staining—Recommended Procedures

Flagella staining is a difficult technic and there have been numerous methods proposed for the purpose. It has long been realized that flagella are actually below the visual limit in size; but of recent years the electron microscope has given a definite idea how small they really are—around 0.02 to 0.03 μ in diameter. Electron micrographs, in fact, indicate that with many kinds of bacteria even the best stained preparations give a very inadequate picture of the actual number or length of the flagella attached to a cell. Were the electron microscope more simple to use, it is possible that it might supplant the light microscope entirely in the demonstration of flagella. Since that is far from the case at present, one must do the best he can with staining methods intended to make the flagella visible. This is usually done by a preliminary mordanting which causes precipitation on the flagella and increases their apparent size—a principle introduced by Loeffler (1890).

A second difficulty in staining flagella is the ease with which bacteria shed these delicate appendages unless the cultures are properly handled. To prevent this one ordinarily employs specially cleaned slides and specially prepared smears on the slides.

Methods for preparing slides. Ordinary cleaning of glassware is not sufficient for the purpose. Various methods have been proposed, but the following directions seem to give as good results as any:

Use new slides if possible preferably of "Pyrex" glass or similar heat resistant properties. (This is because under the drastic method of cleaning to remove grease, old slides have a greater tendency to break.) Clean first in a dichromate cleaning fluid, wash in water and rinse in 95 per cent alcohol; then wipe with a clean piece of cheese cloth. (Wiping is not always necessary but is advisable unless fresh alcohol is used after every few slides.) Pass each slide back and forth through a flame for some time, ordinarily until the appearance of an orange color in the flame; some experience is necessary before the proper amount of heating can be accurately judged.

Unless heat-resistant slides are used, cool slides gradually in order to minimize breakage. An ordinarily satisfactory method of doing this is to place the flamed slides on a metal plate (flamed side up) standing on a vessel of boiling water; and then to remove the flame under the water so as to allow gradual cooling. (Too rapid cooling may result in breakage, sometimes as long as two weeks after the heating.)

Methods of handling cultures. Of various methods proposed, it is not possible to recommend any one as uniformly the best. As any laboratory worker becomes familiar with one particular method, he soon finds he can get better results with that than with any other.

The following method, however, can be given as one of the most satisfactory, especially for students who have not had previous experience with some other method:

Use young and actively growing cultures (e.g. 18–22 hr. old) on agar slants. Before proceding, check the culture for motility in hanging drop. If motile, wash off the growth by gentle agitation with 2–3 ml. sterile distilled water. Transfer to a sterile test tube and incubate at optimum temperature for 10 minutes (30 minutes for those producing slime). At this point, again check motility under a microscope. Transfer a small drop from the top of the suspension (where motile organisms are most numerous), by means of a capillary pipette to one end of the slide prepared as above described. Tilt the slide and allow the drop to run slowly to the other end. (Two or three such streaks can be placed on a slide.) Place the slide in a tilted position and allow it to dry in the air.

Staining procedure. Good results can be obtained with any of the following methods, especially after familiarity has been obtained with it. Special recommendation must be given to the last of the four procedures (modified Bailey method). Although seeming a little more complicated, on first reading, it has been found to give the most uniformly satisfactory results in inexperienced hands.

CASARES-GIL'S FLAGELLA STAIN[3]
AS PUBLISHED BY PLIMMER AND PAINE (1921)

Mordant:

Tannic acid	10 g.
$AlCl_3 \cdot 6H_2O$	18 g.
$ZnCl_2$	10 g.
Basic fuchsin[4]	1.5 g.
Alcohol (60%)	40 ml.

The solids are dissolved in the alcohol by trituration in a mortar, adding 10 ml. of the alcohol first, and the rest slowly. This alcoholic solution may be kept several years. For use, mix with an equal quantity of water (Thatcher, 1926) or dilute with four parts of water (Casares-Gil), filter off precipitate and collect filtrate on the slide.

Staining schedule:

1. Prepare smears of young cultures, on scrupulously cleaned slides as above directed.
2. Filter mordant onto slide as above directed (preferably using Thatcher's 1:1 dilution); allow to act for 60 sec. without heating.
3. Wash in tap water.
4. Flood slide with freshly filtered Ziehl's carbol fuchsin (p. 5), and allow to stand 5 min. without heating.
5. Wash with tap water.
6. Air-dry and examine. Sometimes considerable search may be needed before finding a satisfactorily stained part of the smear.

Results: Fagella well stained (red) in the case of those bacteria (e.g.

[3]See Galli-Valerio (1915).

[4]The authors specify rosanilin hydrochloride. There are, however, other basic fuchsins more universally available which ought to prove equally satisfactory.

colon-typhoid group, aerobic spore-formers) that do not have extremely delicate flagella.

GRAY'S FLAGELLA STAIN
Gray (1926)

Mordant: Solution A

$KAl(SO_4)_2 \cdot 12H_2O$ (sat. aqu. solution) ..... 5 ml.
Tannic acid (20% aqu. solution) ..... 2 ml.
(A few drops of chloroform must be added to this if a large quantity is made up)
$HgCl_2$ (sat. aqu. solution) ..... 2 ml.

Solution B

Basic fuchsin (sat. alc. solution) ..... 0.4 ml.

Mix Solutions A and B less than twenty-four hours before using. Both solutions separately may be kept indefinitely, but deteriorate rapidly after mixing.

Staining schedule:

1. Prepare smears from young cultures as above directed.
2. Flood slide with freshly filtered mordant and allow to act 8–10 min.
3. Wash with a gentle stream of distilled water, and follow steps 4–6 of above schedule (Casares-Gil's method).

Results: Same as with Casares-Gil method.

LEIFSON'S STAIN
Leifson (1930)

$KAl(SO_4)_2 \cdot 12H_2O$, or $NH_4Al(SO_4)_2 \cdot 12H_2O$ (sat. aqu. solution) ..... 20 ml.
Tannic acid (20% aqu. solution) ..... 10 ml.
Distilled water ..... 10 ml.
Ethyl alcohol, 95% ..... 15 ml.
Basic fuchsin (sat. solution in 95% ethyl alcohol) ..... 3 ml.

Mix ingredients in order named. Keep in tightly stoppered bottle and the stain may be good for a week.

Staining schedule:

1. Prepare slides as for the preceding methods.
2. Flood slides with the above solution and allow to stand 10 min. at room temperature in warm weather, or in an incubator in cold weather.
3. Wash with tap water. (If a counterstain is desired, borax methylene blue may be applied, without heat, followed by another washing. See p. IV46-19).
4. Dry and examine.

Results: When no counterstain is used, same as with the two above procedures; with methyelne blue counterstain, see under "Capsule Stains", below.

BAILEY METHOD
Bailey (1929)
MODIFIED BY FISHER AND CONN (1942)

This method is specially recommended for bacteria on which flagella are difficult to stain (as is frequently the case with soil and

water non-spore-formers and with plant pathogens) because of slime production, unusually fine flagella or flagella that are readily lost.

Mordant:

Solution A

Tannic acid (10% aqu. solution)	18 ml.
$FeCl_3 \cdot 6H_2O$ (6% aqu. solution)	6 ml.

Solution B

Solution A	3.5 ml.
Basic fuchsin (0.5% in ethyl alcohol)	0.5 ml.
HCl, concentrated	0.5 ml.
Formalin	2.0 ml.

Staining schedule:

1. Prepare smears of young cultures, following carefully the procedure recommended on p. 15 under "Methods of handling cultures".
2. Filter the above Solution A onto the slide and allow it to remain 3½ min. without heating.
3. Pour off solution A, and without washing add solution B, also through a filter, and allow it to stand 7 min. without heating.
4. Wash with distilled water.
5. Before the slide dries, cover with Ziehl's carbol fuchsin (p. 5), allowing it to stand 1 min. on a hot plate heated just enough for steam to be barely given off.
6. Wash in tap water.
7. Dry in the air and examine.

Results: Similar to the preceding methods; but the background precipitate is usually finer and less conspicuous, thus interfering less with the demonstration of unusually fine, delicate flagella.

Staining flagella of anaerobes. O'Toole (1942) calls attention to certain difficulties in staining the flagella of anaerobes, and gives a modification of the above Bailey stain which is intended to overcome them. The method is not unlike that of Fisher and Conn who had the O'Toole procedure in mind when working out their modification. The O'Toole method does not seem to be as satisfactory as the Fisher and Conn procedure for the above mentioned soil bacteria and plant pathogens; but one must remember that it is particularly recommended by its author for an entirely different type of organism.

Capsule Stains—Recommended Procedures

Bacterial capsules are more easily confused with artifacts than any other structure pertaining to the organisms. Inasmuch as capsules sometimes show merely as unstained areas around the cells, there is a temptation to call any such surrounding area a capsule; very often, however, they merely represent the tendency of a lightly stained surrounding medium to retract from the cells on drying. For this reason the best way to demonstrate capsules is actually to stain them by some procedure which differentiates them from the cell itself. Several of the flagella stains accomplish this, notably those of Bailey and Leifson, given above. Much simpler is the procedure of Anthony described below. The Anthony method can be recommended both because of its simplicity and its dependability. Any of the other

methods which follow give satisfactory results. The student is specially urged, however, not to pronounce any organism capsulated, as a result of any of these staining procedures, until he has carefully compared it with other organisms generally recognized as having capsules.

LEIFSON METHOD
Leifson (1930)

This method is described in detail above (p. 17) and does not need to be repeated here. The special methods of handling slides and cultures, outlined for flagella staining, do not need to be observed, but the following is essential:

After step 3;

4. Stain 5–10 min., without heating, in borax methylene blue (methylene blue, 90% dye content, 0.1 g.; borax 1 g.; distilled water 100 ml.).
5. Wash in tap water.
6. Dry and examine.

Results: capsules red; cells, blue.

ANTHONY'S METHOD WITH TYLER'S MODIFICATION
Anthony (1931)

Original formula		Tyler's modification[5]	
Crystal violet (85% dye content)	1 g.	Crystal violet (85% dye content)	0.1 g.
Distilled water	100 ml.	Glacial acetic acid	0.25 ml.
		Distilled water	100 ml.

Staining schedule:

1. Prepare smears and dry them in the air.
2. Stain 2 min. in the above aqueous crystal violet; or according to Tyler 4–7 min. in the above acetic crystal violet.
3. Wash with 20% aqueous $CuSO_4 \cdot 5H_2O$.
4. Blot dry, and examine.

Results: capsules, blue violet; cells, dark blue.

HISS'S METHOD
Hiss (1905)

ORIGINAL STATEMENT OF FORMULA		EMENDED FORMULA	
Sat. alc. basic fuchsin or gentian violet	5–10 ml.	Basic fuchsin (90% dye content)	0.15–0.3 g.
Water	to make 100 ml.	Distilled water	100 ml.
		or	
		Crystal violet (85% dye content)	0.05–0.1 g.
		Distilled water	100 ml.

Staining schedule:

1. Grow organisms in ascitic fluid or serum medium, or mix with drop of serum and prepare smears from this mixture.
2. Dry smears in the air and fix with heat.
3. Stain with one of the above solutions a few seconds by gently heating until steam rises.

[5]See Park and Williams (1933), p. 84.

4. Wash off with 20% aqueous $CuSO_4 \cdot 5H_2O$.
5. Blot dry, and examine.

Results: capsules, faint blue; cells, dark purple.

Stains for Spirochaetes—Recommended Procedure

Fontana Stain

Preparation of ammoniacal silver nitrate:

Dissolve 5 g. $AgNO_3$ in 100 ml. distilled water. Remove a few milliliters, and to the rest of the solution add drop by drop a concentrated ammonia solution until the sepia precipitate which forms redissolves. Then add drop by drop enough more of the silver nitrate solution to produce a slight cloud which persists after shaking. It should remain in good condition for several months.

Staining schedule:

1. Prepare smear and fix with heat.
2. Pour on a solution of 5% tannic acid in 1% phenol and allow to steam 30 sec.
3. Wash 30 sec. in running water.
4. Cover with a drop of the above ammoniacal silver nitrate, heat gently over a flame and allow it to stand 20–30 sec. after steaming begins.
5. Wash in tap water.
6. Blot dry, and examine.

Results: Spirochaetes, dark brown or black, in a dark maroon field.

Stains for Spirochaetes—Alternate Procedure

Tunnicliff's Stain

Tunnicliff has employed carbol gentian violet (3 to 4 seconds) followed by Lugol's iodine (see p. IV46-8) for the same period in staining bacterial smears. With a slight modification this proves a good spirochaete stain. The modification is:

Carbol crystal violet (1 vol. 10% alc. crystal violet to 10 vol. 1% aq. phenol) 30 seconds; wash with water; the Lugol-Gram iodine solution (see p. IV46-8) 30 seconds; wash with water; safranin 30 seconds; wash with water and dry.

Stain for Rickettsiae

Macchiavello's Method

Staining solution: 0.25 g. basic fuchsin (90% dye content) dissolved in 100 ml. distilled water, buffered to pH 7.2–7.4 with the proper phosphate buffer mixture.

Staining schedule:

1. Smear a bit of tissue on a slide.
2. Dry in the air and fix with gentle heat.
3. Pour the above staining fluid onto the slide through a coarse filter paper. Allow to stand 4 min.
4. Rinse very rapidly with 0.5% aqueous citric acid.
5. Wash quickly and thoroughly with tap water.
6. Counterstain about 10 sec. with 1% aqueous methylene blue.
7. Rinse in tap water.
8. Dry and examine.

Results: Rickettsiae, red; cell nuclei, deep blue; cytoplasm, light blue.

Dye Solubilities at 26°C.

Based on data obtained at the Color Laboratory of the U. S. Dept. of Agriculture. *From Biological Stains by H. J. Conn, 4th Ed. Pub. by Biotech Publications, Geneva, N. Y., 1940.*

Note: These figures are ordinarily for recrystallized dyes. Commercial samples are generally less soluble, often by as much as 30%.

Colour Index Number	Name of dye	Per cent soluble in	
		Water	95% alcohol
1027	Alizarin	nil	0.125
1034	Alizarin red S	7.69	0.15
40	Alizarole orange G	0.40	0.57
36	Alizarole yellow GW	25.84	0.04
184	Amaranth	7.20	0.01
847	Amethyst violet	3.12	3.66
655	Auramin O	0.74	4.49
12	Aurantia	nil	0.33
146	Azo acid yellow	2.17	0.81
88	Azo Bordeaux	3.83	0.19
448	Benzopurpurin 4B	——	0.13
280	Biebrich scarlet	——	0.05
332	Bismarck brown R	1.10	0.98
331	Bismarck brown Y	1.36	1.08
252	Brilliant croceine	5.04	0.06
29	Chromotrope 2R	19.30	0.17
21	Chrysoidin R	0.23	0.99
20	Chrysoidin Y	0.86	2.21
370	Congo red	——	0.19
89	Crystal ponceau	0.80	0.06
681	Crystal violet (chloride) } gentian	1.68	13.87
	Crystal violet (iodide) } violets	0.035	1.78
——	Cresyl violet (N. A. Co.)	0.38	0.25
715	Cyanole extra	1.38	0.44
771	Eosin B (Na salt)	39.11	0.75
768	Eosin Y† (Na salt)	44.20	2.18
	Eosin Y† (Mg salt)	1.43	0.28
	Eosin Y† (Ca salt)	0.24	0.09
	Eosin Y† (Ba salt)	0.18	0.06
130	Erika B	0.64	0.17
254	Erythrin X	6.41	0.06
773	Erythrosin† (Na salt)	11.10	1.87
	Erythrosin† (Mg salt)	0.38	0.52
	Erythrosin† (Ca salt)	0.15	0.35
	Erythrosin† (Ba salt)	0.17	0.04
770	Ethyl eosin	0.03	1.13
——	Fast green FCF	16.04	0.35
176	Fast red A	1.67	0.42
16	Fast yellow	18.40	0.24
766	Fluorescein (color acid)	0.03	2.21
	Fluorescein (Na salt)	50.20	7.19
	Fluorescein (Mg salt)	4.51	0.35
	Fluorescein (Ca salt)	1.13	0.41
	Fluorescein (Ba salt)	6.54	0.56
	Fuchsin, basic:		
676	Pararosanilin (chloride)	0.26	5.93
——	Pararosanilin (acetate)	4.15	13.63
——	Rosanilin (chloride)	0.39	8.16
678	New fuchsin (chloride)	1.13	3.20
——	Gentian violet (see crystal or methyl violet)		

†The color acids of these dyes (not listed here) are practically insoluble in water.

Colour Index Number	Name of dye	Per cent soluble in	
		Water	95% alcohol
666	Guinea green B	28.40*	7.30
1180	Indigo carmine	1.68	0.01
133	Janus green	5.18	1.12
670	Light green SF yellowish	20.35	0.82
657	Malachite green (oxalate)	7.60	7.52
9	Martius yellow, Na salt	4.57	0.16
	Martius yellow, Ca salt	0.05	1.90
138	Metanil yellow	5.36	1.45
142	Methyl orange	0.52	0.08
	Methyl orange (acid)	0.015	0.015
680	Methyl violet (Gentian violet)	2.93	15.21*
922	Methylene blue ($ZnCl_2$ double salt)	2.75	0.05
	Methylene blue (chloride)	3.55	1.48
	Methylene blue (iodide)	0.09	0.13
924	Methylene green	1.46	0.12
10	Naphthol yellow G	8.96	0.025
152	Narcein	10.02	0.06
825	Neutral red (chloride)	5.64	2.45
	Neutral red (iodide)	0.15	0.16
826	Neutral violet	3.27	2.22
927	New methylene blue N	13.32*	1.65
728	New Victoria blue R	0.54	3.98
520	Niagara blue 4B	13.51	nil
914	Nile blue 2B	0.16	0.62
73	Oil red O	nil	0.39
150	Orange I	5.17	0.64
151	Orange II	11.37	0.15
27	Orange G	10.86	0.22
714	Patent blue A	8.40	5.23
774	Phloxine† (Na salt)	50.90*	9.02
	Phloxine† (Mg salt)	20.84	29.10
	Phloxine† (Ca salt)	3.57	0.45
	Phloxine† (Ba salt)	6.01	1.17
7	Picric acid	1.18	8.96
28	Ponceau 2G	1.75	0.21
186	Ponceau 6R	12.98	0.01
741	Pyronin B (iodide)	0.07	1.08
739	Pyronin Y	8.96	0.60
148	Resorcin yellow	0.37	0.19
749	Rhodamine B	0.78	1.47
750	Rhodamine G	1.34	6.31
779	Rose bengal† (Na salt)	36.25	7.53
	Rose bengal† (Mg salt)	0.48	1.59
	Rose bengal† (Ca salt)	0.20	0.07
	Rose bengal† (Ba salt)	0.17	0.05
841	Safranin	5.45	3.41
689	Spirit blue	nil	1.10
24	Sudan I	nil	0.37
248	Sudan III	nil	0.15
258	Sudan IV	nil	0.09
920	Thionin	0.25	0.25
925	Toluidine blue O	3.82	0.57
690	Victoria blue 4R	3.23	20.49
659	Victoria green 3B	0.04	2.24
8	Victoria yellow	1.66	1.18

*These figures are grams per hundred grams of saturated solution (the others being grams per hundred milliliters).

†The color acids of these dyes (not listed here) are practically insoluble in water.

REFERENCES

ALBERT, HENRY. 1920. Diphtheria bacillus stains with a description of a "new" one. Am. J. Pub. Health, **10**, 334–7.

————. 1921. Modification of stain for diphtheria bacilli. J. Amer. Med. Assn., **76**, 240.

ANTHONY, E. E. 1931. A note on capsule staining. Science, **73**, 319.

BAILEY, H. D. 1929. A flagella and capsule stain for bacteria. Proc. Soc. Exp. Biol. & Med., **27**, 111–2.

BARTHOLOMEW, J. W., and UMBREIT, W. W. 1944. Ribonucleic acid and the Gram stain. J. Bact., **48**, 567–78.

BENIANS, T. H. C. 1916. Relief staining for bacteria and spirochaetes. Brit. Med. J. **1916** (2). 722.

BLUMENTHAL, J. M. and LIPSKEROW, M. 1905. Vergleichende Bewertung der differentiellen Methode zur Färbung des Diphtheriebacillus. Centbl. f. Bakt. I Abt., Orig., **38**, 359–66.

BURKE, VICTOR. 1921. The Gram stain in the diagnosis of chronic gonorrhea. J. Amer. Med. Assoc., **77**, 1020–2.

BURKE, VICTOR. 1922. Notes on the Gram stain with description of a new method. J. Bact., **7**, 159–82.

CONKLIN, MARIE E. 1934. Mercurochrome as a bacteriological stain. J. Bact., **27**, 30.

CONN, H. J. 1946. Biological Stains. 5th Ed. Biotech Publications, Geneva, N. Y.

CONN and DARROW. 1943–5. Staining Procedures. Biotech Publications, Geneva, N. Y.

COOPER, F. B. 1926. A modification of the Ziehl-Neelsen staining method for tubercle bacilli. Arch. Path. & Lab. Med., **2**, 382–5.

DORNER, W. C. 1922. Ein neues Verfahren für isolierte Sporenfärbung. Landw. Jahrb. d. Schweiz., **36**, 595–7.

DORNER, W. C. 1926. Un procédé simple pour la coloration des spores. Le Lait, **6**, 8–12.

DORNER, W. C. 1930. The negative staining of bacteria. Stain Techn., **5**, 25–7.

FISHER, P. J. and CONN, JEAN E. 1942. A flagella staining technic for soil bacteria. Stain Techn., **17**, 117–21.

FONTANA, ARTUR. 1912. Verfahren zur intensiver und rascheu Färbung des Treponema pallidum und anderer Spirochäten. Derm. Wochnsch., **55**, 1003–4.

GALLI-VALERIO, B. 1915. La méthode de Casares-Gil pour la coloration des cils des bactéries. Centbl. f. Bakt., I Abt. Orig., **76**, 233–4.

GRAY, P. H. H. 1926. A method of staining bacterial flagella. J. Bact., **12**, 273–4.

HENRY, H. and STACEY, M. 1943. Histochemistry of the Gram-staining reaction for micro-organisms. Nature, **151**, 671.

HISS, P. H., JR. 1905. A contribution to the physiological differentiation of Pneumococcus and Streptococcus, and to methods of staining capsules. J. Exp. Med., **6**, 317–45.

HUCKER, G. J. 1922. Comparison of various methods of Gram staining. (Preliminary Report.) Abstr. Bact., **6**, 2.

HUCKER, G. J. and CONN, H. J. 1923. Methods of Gram staining. N. Y. S. Agr. Exp. Sta., Tech. Bul. 129.

HUCKER, G. J. and CONN, H. J. 1927. Further studies on the methods of Gram staining. N. Y. S. Agr. Exp. Sta., Tech. Bul. 128.

JOHNSTON, O. P., and MACK, W. B. 1904. A modification of existing methods for staining flagella. American Medicine, **7**, 754.

KINYOUN, J. J. 1915. A modification of Ponder's stain for diphtheria. Am. J. Pub. Health, **5**, 246–7.

KOPELOFF, N. and BEERMAN, P. 1922. Modified Gram stains. J. Inf. Dis., **31**, 480–2.

LAYBOURN, R. L. 1924. A modification of Albert's stain for the diphtheria bacilli J. Amer. Med. Assn., **83**, 121.

LEIFSON, EINAR. 1930. A method of staining bacterial flagella and capsules together with a study of the origin of flagella. J. Bact., **20**, 203–11.

LOEFFLER, F. 1884. Untersuchungen über die Bedeutung der Mikroorganismen fur die Entstehung der Diphtherie beim Menschen, bei der Taube und beim Kalbe. Mitt. a.d.k. Gesundheitsamte, **2**, 421–99.

See p. 439.

MALLORY, F. B. and WRIGHT, J. H. 1924. Pathological Technique. *5th Ed.* Saunders, Philadelphia.

MUCH, H. 1907. Über die granuläre, nach Teil nicht färbbare Form des Tuberkulosevirus. Beitr. z. Klin. d. Tuberel., **8**, 85–99.

NEELSEN, F. 1883. Ein casuistischer Beitrag zur Lehre von der Tuberkulose. Centbl. f. Med. Wissensch., **21**, 497–501. (See p. 500.)

NEISSER, M. 1903. Die Untersuchung auf Diphtheriebaeillen in centralisierten Untersuchungsstationen. Hyg. Rundsch., **13**, 705–17.

NICOLLE, CH. 1895. Pratique des colorations microbiennes. Ann. Inst. Past., **9**, 664–70.

O'TOOLE, Elizabeth. 1942. Flagella staining of anaerobic baeilli. Stain Techn., **17**, 33–40.

PARK, W. H. and WILLIAMS, ANNA W. 1933. Pathogenic Microorganisms. 10th Ed. Lea & Febiger, Philadelphia.

PLIMMER, H. G., and PAINE, S. G. 1921. A new method for the staining of bacterial flagella. J. Path. & Bact., **24**, 286–8.

PONDER, C. 1912. The examination of diphtheria specimens. A new technique in staining with methylene blue. Lancet, **2**, 22–3.

RICHARDS, O. W. and MILLER, D. K. 1941. An efficient method for the identification of M. tubereulosis with a simple fluorescence microscope. Amer. J. Clin. Path., **11**, 1–7.

SCHAEFFER, ALICE B. and FULTON, McD. 1933. A simplified method of staining endospores. Science, **77**, 194.

SNYDER, MARION A. 1934. A modification of the Dorner spore stain. Stain Techn., **9**, 71.

THATCHER, LIDA M. 1926. A modification of the Casares-Gil flagella stain. Stain Techn., **1**, 143.

TUNNICLIFF, RUTH. 1922. A simple method of staining Gram-negative organisms. J. Am. Med. Assn., **78**, 191.

WEISS, L. 1909. Zur Morphologie des Tuberkulosevirus unter besonderer Berücksichtigung einer Doppelfärbung. Berl. Klin. Wochensch. **46**, 1797–800.

WIRTZ, R. 1908. Ein einfache Art der Sporenfärbung. Centbl. f. Bakt., I Abt. Orig., **46**, 727–8.

ZIEHL, F. 1882. Zur Färbung des Tuberkelbaeillus. Deut. Med. Wehnsch., **8**, 451.

LEAFLET V

ROUTINE TESTS FOR THE DESCRIPTIVE CHART

MORPHOLOGICAL AND BIOCHEMICAL

by

H. J. Conn

11th EDITION

Pure Culture Study of Bacteria, Vol. 17, No. 3–4

September, 1949

Revised with the assistance of

M. W. Jennison, L. S. McClung, C. A. Stuart and C. E. ZoBell

LEAFLET V

ROUTINE TESTS FOR THE DESCRIPTIVE CHART

MORPHOLOGICAL AND BIOCHEMICAL

Introduction

The Society of American Bacteriologists issues Descriptive Charts for use in characterizing bacterial species. The Charts are blank forms on which the characteristics of any culture under investigation are to be recorded, at least one chart to be used for each culture studied. The Manual of Methods for Pure Culture Study of Bacteria was originally published to secure uniformity in the methods used for determining these characteristics. At the present time the scope of the Manual has become much broader than this, and practically all the methods covered by the original Manual are now included in this leaflet.

The methods described in this leaflet are intended primarily for aerobic saprophytes, and cannot therefore be considered applicable in general to obligate parasites or strict anaerobes. Leaflet III must be consulted in studying the latter group; while Leaflet VII gives methods specially applicable to animal pathogens. Special methods for plant pathogens are given in Leaflet X. In the case of other special groups, the investigator will often find the methods given here to be unsatisfactory and will therefore be forced to modify them or to use others more suited to the group in question.

The Descriptive Charts

There are two Descriptive Charts, each printed on 8½ by 11 inch sheets of heavy paper: the Standard Descriptive Chart, and the Descriptive Chart for Instruction. The general plan of each is to have the body of it consist, under various headings, of a series of blanks to be completed and descriptive terms to be underlined, as the various characteristics of the cultures are determined. In addition to this, there is a place on the margin for recording the most important characteristics by a system of numerical notation.

The special feature of the Standard Descriptive Chart is that all the most important characteristics of an organism may be recorded on the front of the sheet, partly in the margin, partly in the larger section at the right, while the fermentative reactions are to be

entered at the bottom. By the use of right-hand margin and bottom edge, a long series of charts may be compared, one on top of the other, by glancing only at these two edges. The back of the Standard Chart is now reserved largely for supplementary data, nearly all of which is summarized on the front. (See first insert, following p. 12.)

The increasingly large number of tests called for in the study of bacteria has resulted in making a somewhat complicated chart. Although all these tests may be needed in some research work, they plainly are not needed in the use of the chart for instruction purposes. To meet the demand for a simpler chart for use in teaching, a new form known as the Descriptive Chart for Instruction was published in 1939. This chart is designed to fit a standard note-book for 11 by 8½ inch sheets. (See second insert, p 12.) In numerous research laboratories, also, this chart is proving more useful than the Standard Chart, because of its flexibility and the amount of space available for special tests.

Determining Optimum Conditions for Growth

Before beginning the study of any pure culture, it is important to know something about the growth requirements of the organism. If the organism in question does not grow in ordinary media, either because it is an obligate parasite or because it requires the complete absence of oxygen or of organic matter, it obviously cannot be studied by the methods called for on the Descriptive Chart. For such organisms the investigator must use his own methods of study, and may record the results in the blank space at the bottom of the back of the Chart. For those organisms that grow on ordinary media, methods must be varied according to whether the organisms grow better in liquid or in solid media and at high temperature or low temperature. It is important, therefore, that before studying an unknown culture which is able to grow in laboratory media, these two points in regard to growth requirements be determined. (As pointed out in Leaflet II, many such media are now available in dehydrated form.)

After these growth peculiarities are determined, it is possible to proceed with the study of an organism under optimum conditions. Space is left on the Chart under all of the procedures listed where the medium used and the temperature of incubation can be recorded. So far as possible the same uniform set of conditions should be used throughout the entire study of one organism. If, for example, one set of tests is made on solid media at 25°C, the other tests should be made likewise. Leaving out those organisms referred to above which require special conditions of study, and other organisms of peculiar growth requirements, such as the thermophilic bacteria, there are four different sets of conditions that will suit practically all bacteria,

namely: liquid media at 37°; solid media at 37°; liquid media at 21–25°; and solid media at 21–25°.

Space is provided on the Standard Chart for recording optimum medium and temperature. This does not ordinarily mean that one must determine the one best medium for the growth of the culture nor the exact degree of temperature at which it grows most rapidly. In the first blank one may record such terms as "organic, solid," "organic, liquid," "inorganic, solid" etc., unless it be known that there is one particular medium specially adapted to the organism in question. Under the second blank one may record temperature in general terms, as: "20–25°", "35–40°", "45–50°", or "over 55°."

It is also important to remember that certain organisms (frequently facultative anaerobes) which do not grow in either solid or true liquid media, will grow in a "semi-solid" medium (that is a nutrient solution in which 0.05–0.1% of agar has been dissolved). It is of course important that such organisms be studied under optimum conditions; and for their study the procedures given in this Manual should ordinarily be modified by using media containing 0.05–0.1% agar instead of the usual liquid or solid media.

Thermal death point, as called for under "Temperature Relations" on the back of the Chart, is undoubtedly best determined with the use of capillary tubes. Short pieces of thin-walled tubing having an internal diameter of 1–1.5 mm. are filled with the culture (consisting mostly of spores, if it is a spore-former) and are heated for varying periods of time at the temperatures under investigation. After heating, each tube is broken into a tube of a medium in which the organisms grow well. A tabulation of results gives a good idea of the thermal death point. This procedure requires careful attention to detail; and one should consult the description of it by Magoon (1926). Results are most valuable if the length of time before death is recorded: in which case, this becomes a test for *Thermal death time.*

Incubation

Cultures should be incubated at or near the optimum temperature of the organism or organisms under investigation. As a rule it is not necessary, however, to know the exact optimum temperature of each organism. If the laboratory is equipped with a series of incubators running at 20°, 25°, 30°, and 37°C, the temperature requirements of practically all bacteria except the thermophilic forms can be very satisfactorily met. Room temperature is sometimes used in place of 25°; but is not to be recommended because of its uncontrollable variations.

Length of incubation varies and is specified on the Chart under many of the tests. In cases where it is not specified one should observe the following general rule: On the day when good growth

first appears the proper descriptive terms on the Chart should be underlined. Any changes occurring and noted in subsequent study should also be recorded on the Chart. The meaning of the terms given in this section of the Chart will in general be made clear by consulting the glossary included in Leaflet I.

Variation

In using these methods it must be remembered that among bacteria, the individual members of any species may differ from each other in respect to both physiology and morphology, thus making it difficult to define the limits of the species; also that any individual culture in repeated examinations may produce variable results in connection with some test even when studied under apparently constant conditions. For these reasons it is important that single determinations shall never be used for characterizing any culture that has been studied, or much less for characterizing any species or type that is being described. Determinations must be repeated at different times and under different conditions in order to learn definitely the physiological characteristics of a culture. Whenever possible, an effort should be made to correlate the variations in physiology and serology with colony type and to list separately the physiological characteristics of the "smooth", "rough", "mucoid", "opaque", "translucent" strains, etc. When an organism shows any tendency to "dissociate" into "phase variants", its description is incomplete if it applies to only one phase or to a culture containing a mixture of two phases or more. In such case the phase variants should be separated by plating methods, or otherwise, and a separate chart should be used for each individual strain studied. The individual charts may be filed for the investigator's information; but it must be insisted that results of such work should not be published for the use of other bacteriologists until repeated determinations have been made and, if possible, have been shown to bear some relation to the phase indicated by colony type.

Cultural Characteristics

Space is provided on both Charts for recording appearance of colonies, growth on agar stroke, in broth and gelatin stab. In addition to the space provided for sketches, various terms are listed in order that those which apply may be underlined. The meaning of all the terms is given in the Glossary in Leaflet I.

As some of the terms, especially in regard to shape and structure of colonies, are more easily described graphically than verbally the diagram on page 7 (also published separately) is included here to assist the student in understanding the appropriate terms.

Study of Cell Morphology

The routine study of morphology should include examinations of stained dried preparations and of unstained organisms in hanging drop. Stained preparations to show the vegetative cells should be made, preferably from agar slant cultures, from a few hours to two days old, according to the rapidity of growth. The medium and temperature used and the age of the culture should be recorded. The examination of unstained organisms in hanging drop is a useful supplementary procedure too often neglected.

Motility. Hanging-drop preparations of young broth or agar cultures should be examined for motility. Before drawing definite conclusions, cultures grown at several temperatures between 20° and 37°C. should be examined. It is important not to confuse Brownian or molecular movement with true motility. The former consists of a "to and fro" motion without change in position, except as influenced by currents in the fluid. A phase microscope can prove useful in studying motility.

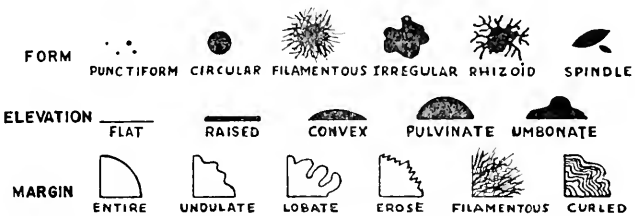
When interpreting results it is important to remember that whereas definite motility in a hanging drop preparation is conclusive, weak motility or none has little significance, and other means of confirmation, such as those that follow, must be undertaken. In particular, an increasing number of cases are found of organisms fully flagellated as shown by staining methods and serology, but absolutely nonmotile by any other method—bacteria with so-called "paralyzed flagella".

Tittsler and Sandholzer (1936) have, in fact, proposed the use of stabs in a semi-solid agar (meat extract 0.3%, peptone 0.5%, agar 0.5%). Motile organisms show a diffuse zone of growth spreading from the line of inoculation; non-motile cultures do not. For this test, incubation should be for 6 days at 30°C. unless positive results are secured sooner. For Gram-negative non-spore-formers, 12–18 hour incubation gives more clear-cut results. This test is a good check on the hanging drop method, but is slow and requires some experience before one can be certain how to interpret results.

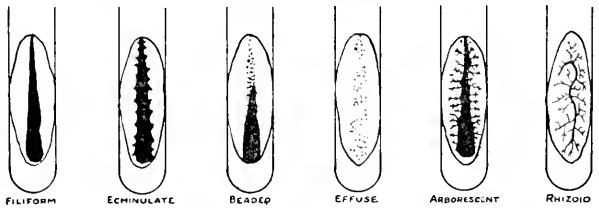
For this reason Conn and Wolfe (1938) have recommended a flagella stain even on cultures that do not appear motile upon examination in hanging drop. The modification of the Bailey flagella stain given in Leaflet IV is simple and quick enough to be employed for routine examinations; positive results cannot be misinterpreted, and show the arrangement of flagella as well as the mere presence or absence of motility. A few further refinements of the method, mak-

CULTURAL CHARACTERISTICS OF BACTERIA

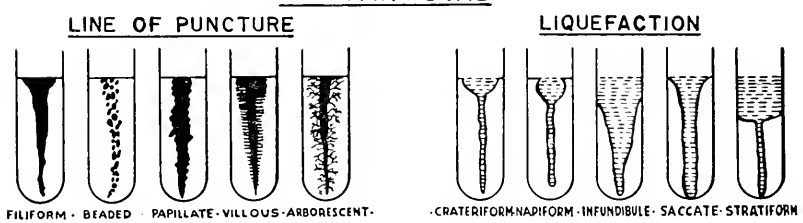
COLONIES



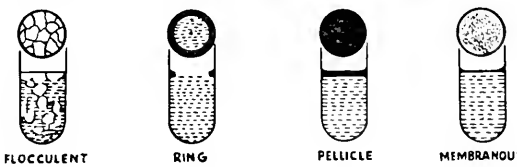
AGAR STROKE - FORM OF GROWTH



GELATIN STAB



NUTRIENT BROTH - SURFACE GROWTH



(Copies of this chart on sale by Biotech Publications, Geneva, N. Y.)

ing it more adaptable to routine use on bacteria of various types, published by Fisher and Conn (1942), is also given in Leaflet IV.

Presence of endospores. Routine examinations should be made on agar slant cultures a week old, employing methylene blue or dilute crystal violet, to stain the vegetative rods and leave spores unstained. If spore-like bodies are present whose exact nature is uncertain, one of the spore stains recommended in Leaflet IV should be employed.

In most cases there is little trouble in finding spores if the organism produces them. All rather large rods, however, (0.8 micron or more in diameter) should be regarded as possible spore-producers even if microscopic examination does not show spores. Such bacteria should be mixed with sterile broth or physiological saline solution and heated to 85°C. for ten minutes; if still alive, endospores may be regarded as probably present. One should also make repeated transfers of the culture onto agar and examine at various ages. A culture of a large rod should not be recorded as a non-spore-former unless all these tests are negative.

Acid-fast staining. Various methods have been proposed for determining whether an organism is "acid fast." They are all essentially modifications of the same general procedure, and are similar to the spore stains of Moeller (1891) and Foth (1892). The Committee is as yet unprepared to recommend any one of them in particular. Several are listed in Leaflet IV.

Capsules. An organism should not be recorded as having capsules unless they have been actually stained by one of the methods of capsule staining described in bacteriological text books. Four of the common methods of capsule staining, namely those of Anthony, of Hiss, of Huntoon, and of Churchman, are given in Leaflet IV. The Committee has obtained good results with Anthony's and Hiss' methods. Capsules do not appear in all media; the medium of choice should be milk serum slants, or exudates from infected animals.

Irregular forms. Forms that differ from the typical shape for the organism, such as branching forms, clubs, spindles, or filaments should be noted and sketched. Simple observation is enough to show that these irregular forms occur quite uniformly in certain cultures, hence their existence must not be ignored; the *interpretation* of these forms is at present under dispute and the decision as to their significance must be awaited. The Committee recommends that the microscopic study of any culture include an examination of the growth on various media and at various ages upon each medium, with sketches of all the shapes that occur.

Gram stain. The Gram stain was until recently an entirely empirical procedure for distinguishing between two groups of organisms, the actual significance of which was not understood. Since 1940, however, the work of Henry and Stacey (1943), of Bartholomew and Umbreit (1944) and others has shown that a positive reaction is dependent upon the presence of ribonucleic acid in the outer layers of the cells, which can be removed by treatment with ribonuclease and replated on them by treatment with magnesium ribonucleate. Thus Gram-positive organisms can be artificially converted to Gram-negative ones and then restored to their Gram-positive state.

In addition to this fact, it is also true that many bacteria are neither definitely positive nor negative; some organisms are Gram-variable and may appear either negative or positive according to conditions. Other organisms contain granules which resist decolorization and may cause misinterpretation. The importance of taking such variations into account has been emphasized in all previous editions of this Leaflet. (Also see Committee Report, 1927.) Such organisms should be recorded as Gram-variable rather than made to appear either positive or negative by some modification of technic. To determine whether an organism belongs to this variable group, it is necessary that it be stained at two or three different ages by more than one procedure. If an organism changes from positive to negative or vice versa during its life history, this change should be recorded, with a statement as to the age of the culture when the change was first observed. It is often practical to record such an organism as prevailingly positive or prevailingly negative; obviously, however, this cannot be done without a very considerable series of determinations. Tests must therefore be made after 1 day's and 2 days' incubation, sometimes also in even older cultures. It must, moreover, be recognized that *Gram-variable* organisms are not necessarily ones that show *uneven* Gram staining; the latter should be recorded as staining unevenly, not as Gram-variable.

The two methods at present recommended are the ammonium oxalate method (Hucker) and Kopeloff and Beerman's modification of the Burke technic. In the former the manipulation is more simple; but the latter is understood to give better results if the organism is growing in a medium that may be of acid reaction, and is claimed to distinguish better between true and false positive reactions. These two procedures are given in Leaflet IV.

Relation to Free Oxygen

In relation to free oxygen, organisms are generally classified as strict aerobes, facultative anaerobes, or strict anaerobes. A fourth group of microaerophiles may also be recognized. None of these distinctions is clear-cut; but the following method gives a rough grouping of bacteria in regard to their oxygen requirements.

Agar shake culture affords a good routine method of determining the oxygen requirements of an organism. A tube of deep agar medium containing glucose or some other available carbon source, is inoculated while in fluid condition at 45°C. with an inoculum not too heavy to permit discrete colonies, rotated to mix the inoculum with the medium, and cooled. Some bacteriologists prefer to pour or pipet the inoculated medium into another sterile tube to insure thorough mixing.

Upon incubation, strict aerobes will be found to grow upon the surface and in the upper layers only; microaerophiles will grow best just a few millimeters below the surface; facultative anaerobes will grow throughout the medium; and strict anaerobes will grow only in the depths, if at all.

Action on Nitrates

Nitrate reduction should be indicated by complete or partial disappearance of nitrate accompanied by appearance of nitrite, ammonia, or free nitrogen. As quantitative nitrate tests are too time-consuming for routine pure culture work, one must ordinarily be satisfied with tests for the end-products only.

The following routine procedure is recommended: Inoculate into nitrate broth and onto slants of nitrate agar (containing 0.1% KNO_3 plus beef extract and peptone as usual). Test the cultures on various days as indicated on the Chart. On these days examine first for gas as shown by foam on the broth or by cracks in the agar. Then test for nitrite with the following reagents.

1. Dissolve 8 grams sulphanilic acid in 1 liter of 5 N acetic acid (1 part glacial acetic acid to 2.5 parts of water), or in 1 liter of dilute sulphuric acid (1 part concentrated acid to 20 parts water).

2. Dissolve 5 grams *a*-naphthylamine in 1 liter of 5 N acetic acid or of very dilute sulphuric acid (1 part concentrated acid to 125 parts water). Or dissolve 6 ml. of dimethyl-*a*-naphthylamine in 1 liter of 5 N acetic acid. This latter reagent has recently been recommended by Wallace and Neave (1927), and by Tittsler (1930) as it gives a permanent red color in the presence of high concentrations of nitrite.

Put a few drops of each of these reagents in each broth culture to be tested, and on the surface of each agar slant. A distinct pink or red in the broth or agar indicates the presence of nitrite. It is well

to test a sterile control which has been kept under the same condition to guard against errors due to absorption of nitrous acid from the air.

Presence of nitrite shows the nitrate to have been reduced, and the presence of gas is a strong indication that reduction has taken place. A negative result does not prove that the organism is unable to reduce nitrates; in such a case further study is necessary as follows:

In case the fault seems to lie in poor growth, search should be made for a nitrate medium in which the organism in question does make good growth by means of the following modifications: increasing or decreasing the amount of peptone; changing the amount of nitrate; altering the reaction; adding some readily available carbohydrate; adding 0.1–0.5% agar to a liquid medium to furnish a semi-solid substrate. The appearance of nitrite in any nitrate medium whatever (while it is absent in a sterile control) should be recorded as nitrate reduction.

Absence of nitrite in the presence of good growth may indicate complete consumption of nitrate or its decomposition beyond the nitrite stage as well as *no* reduction at all. Test, therefore, for nitrate by adding a pinch of zinc dust to the tube to which the nitrite reagents have been introduced and allowing it to stand a few minutes. If nitrate is present it will be reduced to nitrite and show the characteristic pink color. Confirmation of the test may be obtained by placing a crystal of diphenylamine in a drop of concentrated sulfuric acid in a depression in a porcelain spot plate and touching with a drop of the culture (or of the liquid at the base of the slant if agar cultures are used). The test will be more delicate if the culture is first mixed with concentrated sulfuric acid and allowed to cool. A blue color indicates presence of nitrate, provided nitrite is absent; but as nitrite gives the same color with diphenylamine, this test must not be used when nitrite is present in the same or greater order of magnitude.

If none of these tests indicate utilization of the nitrate, the organism probably does not reduce nitrate, but to be certain of the fact further investigation is necessary as outlined in Leaflet VI. It must be understood, however, that for routine diagnostic work a determination of nitrite on standard nitrate broth or agar is ordinarily sufficient; this is because most descriptions in the literature containing the words "Nitrates not reduced" merely mean that no nitrite is produced on this medium. *But in recording such results the student should be careful to state only the observed fact, i.e. that nitrite is or is not found in the nitrate medium employed.*

Chromogenesis

Color production should be recorded if observed in broth, on beef-extract agar, gelatin or potato, or if noticed to a striking extent on any other medium (*e.g.*, starch media). In the margin the space devoted to chromogenesis refers to the color produced on beef extract agar. Note differences, if any, in pigmentation of growth exposed to air and shielded from air, or in presence or absence of light. Frequently it is well to note the final H-ion concentration of the culture, as some pigments act as H-ion indicators.

Indole Production

During the last 40 years, results of investigations on the indole test have been published by Zipfel (1912), Frieber (1921), Fellers and Clough (1925), Goré (1921), Holman and Gonzales (1923), Kulp (1925), Koser and Galt (1926) and Kovács (1928). The two important points brought out in these papers are: that the medium be of correct composition; and that the test used be specific for indole.

The important consideration in regard to the medium is that a peptone be employed containing tryptophane, which is not always present in bacteriologic peptones. Peptones are ordinarily digests of lean meat; but for the indole test a casein digest which contains tryptophane is apparently more satisfactory.

The medium used should, therefore, contain 1.0% of casein digest. No other ingredients need be added if the organism under investigation will grow in a solution of it alone. If the organism is not able to grow in such a medium, add such ingredients as are needed to assure its growth. If necessary, add agar and perform the test on agar slants.

If the organism produces good growth, 1–2 days' incubation is ordinarily sufficient. In fact, with rapid-growing organisms, the reaction may be positive in 24 hours, but negative the following day. Therefore both 24-hour and 48-hour tests are recommended. The test for indole may be performed by the technic of Ehrlich-Böhme, by either the Goré or the Kovács modification of the same, or by the Gnezda technic. The Kovács method is especially simple and convenient. These procedures are as follows:

Böhme (1905) called for the following solutions:

Solution 1

Para-dimethyl-amino-benzaldehyde	1 g.
Ethyl alcohol (95%)	95 ml.
Hydrochloric acid, concentrated	20 ml.

Solution 2

Saturated aqueous solution of potassium persulfate ($K_2S_2O_8$).

To about 10 ml. of the culture fluid add 5 ml. of solution No. 1, then 5 ml. of solution No. 2, and shake; a red color appearing in five minutes indicates a positive reaction. This test may also be performed (and sometimes more satisfactorily) by first shaking up the culture with ether and adding solution No. 1 (Ehrlich's reagent) dropping down the side of the tube so that it spreads out as a layer between the ether and the culture fluid. After this method of applying, solution No. 2 seems to be unnecessary.

The Goré (1921) test uses these same solutions, but the method of application is as follows: Remove the plug of the culture tube (which must be of white *absorbent* cotton), moisten it first with four to six drops of solution No. 2, then with the same

STANDARD DESCRIPTIVE CHART

Name of organism ..... Source ..... Studied by ..... Culture No. .....

Date of isolation ..... Habitat ..... Optimum conditions: Media ..... Temp. ..... °C.

Is phase variation observed? ..... Phase on this Chart: S, R, M, G (smooth, rough, mucoid, gonidial) ..... Phases recorded on other charts: .....

Underscore required terms.

VEGETATIVE CELLS: Medium used .....
Reaction (pH) ..... Temp. ..... Age ..... d
Size of Majority .....
Ends, *rounded, truncate, concave, tapering*
MOTILITY: In broth ..... On agar .....
SPORANGIA and ENDOSPORES: *present, absent.*
Medium used ..... pH ..... Temp. ..... Age ..... d
Endospore Form: *spherical, ellipsoid, cylindrical*
IRREGULAR FORMS:
Present on ..... in ..... days at ..... °C.

SKETCHES

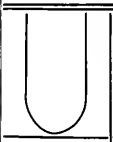
AGAR COLONIES: Temperature ..... °C. Age ..... d.
Form, *punctiform (i. e. under 1 m.m. diam.), circular (i. e. over 1 m.m. diam.), filamentous, irregular, rhizoid.*
Surface, *smooth, rough, concentrically ringed, radiately ridged.*
Edge, *entire, undulate, lobate, erose, filamentous, curled.*
Elevation of growth, *effuse, flat, raised, convex.*
Optical Characters, *opaque, translucent, opalescent, iridescent.*

Surface Colonies | Deep Colonies

GELATIN COLONIES: Temperature ..... °C. Age ..... d.
Form, *punctiform, circular, irregular, filamentous.*
Elevation, *flat, raised, convex, pulvinate, crateriform (liquefying).*
Edge, *entire, undulate, lobate, erose, filamentous, curled.*
Liquefaction, *cup, saucer, spreading.*
Surface, *smooth, contoured, rugose.*
Optical Characters, *opaque, translucent, opalescent, iridescent.*

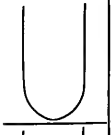
Surface Colonies | Deep Colonies

AGAR STROKE: Temperature ..... °C. Age ..... d.
Growth, *scanty, moderate, abundant, none.*
Form of growth, *filiform, echinulate, beaded, spreading, arborescent, rhizoid.*
Lustre, *glistening, dull.*
Chromogenesis ..... *photogenic, fluorescent.*
Odor, *absent, decided, resembling* .....
Consistency, *butyrous, viscid, membranous, brittle.*
Medium, *grayed, browned, reddened, blued, greened, unchanged.*



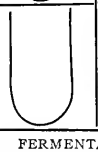
Medium: Temperature ..... °C. Age ..... d.

NUTRIENT BROTH: Temperature ..... °C. Age ..... d.
Surface growth, *ring, pellicle, flocculent, membranous, none.*
Clouding, *slight, moderate, strong, transient, persistent, none, fluid turbid, granular growth.*
Odor, *absent, decided, resembling* .....
Sediment, *compact, flocculent, granular, flaky, viscid.*
Amount of sediment, *abundant, scanty, none.*



Medium: Temperature ..... °C. Age ..... d.

GELATIN STAB: Temperature ..... °C. Age ..... d.
Growth, *uniform, best at top, best at bottom.*
Line of puncture, *filiform, beaded, papillate, villous, arborescent.*
Liquefaction, *none, crateriform, infundibuliform, napiform, saccate, stratiform:* begins in ..... d. complete in ..... d.
Degree of liquefaction in ..... days .....
Method used .....
Medium, *fluorescent, browned, unchanged.*



Medium: Temperature ..... °C. Age ..... d.

FERMENTATION Temperature ..... °C.

Medium containing ..... and:	Monosaccharides							Disaccharides							Polysaccharides							Alcohols								Glucosides				
	Arabinose	Rhamnose	Xylose	Glucose	Fructose	Galactose	Mannose	Lactose	Sucrose	Maltose	Trehalose	Melibiose	Cellobiose		Raffinose	Melezitose	Starch	Inulin	Dextrin	Glycogen		Glycerol	Erythritol	Arabitol	Adonitol	Mannitol	Sorbitol	Dulcitol		Salicin	Aesculin	Coniferin	a-Methyl Gluc.	
Gas in fermentation tube																																	
Amt. CO_2 in Eldredge tube																																	
Reaction (pH) after ..... d.																																	
Titrable acidity in ml. of N/..... NaOH																																	

BRIEF CHARACTERIZATION

As each of the following characteristics is determined, indicate in proper marginal square by means of figure, as designated below. In case any of these characteristics are doubtful or have not been determined, indicate with the letters U, V, and X according to the following code:

U, undetermined; V, variable; X, doubtful.

MORPHOLOGICAL

VEGETATIVE CELLS

- Form & arrangement: 1, streptococci; 2, diplococci; 3, micrococci; 4, sarcinæ; 5, rods; 6, commas; 7, spirals; 8, branched rods; 9, filamentous
- Diameter: 1, under 0.5μ; 2, between 0.5μ and 1μ; 3, over 1μ
- Gram stain: 0, negative; 1, positive
- Flagella: 0, absent; 1, peritrichic; 2, polar; 3, present but undetermined
- Capsules: 0, absent; 1, present
- Chains (4 or more cells): 0, absent; 1, present

SPORANGIA: 0, absent; 1, elliptical; 2, short rods: 3, spindled; 4, clavate; 5, drumsticks

ENDOSPORES: 0, absent; 1, central to excentric; 2, subterminal; 3, terminal

CULTURAL

AGAR STROKE
- Growth: 0, absent; 1, abundant; 2, moderate; 3, scanty
- Lustre: 1, glistening; 2, dull

AGAR COLONIES
- Form: 1, punctiform; 2, circular (over 1 mm. diameter); 3, rhizoid; 4, filamentous; 5, curled; 6, irregular
- Surface: 1, smooth; 2, contoured; 3. rugose

GELATIN COLONIES
- Form: 1, punctiform; 2, circular (over 1 mm.); 3, irregular; 4, filamentous
- Surface: 1, smooth; 2, contoured; 3, rugose

PHYSIOLOGICAL

- Biologic relationships: 1, pathogenic for man; 2, for animals but not for man; 3, for plants; 4, parasitic but not pathogenic; 5, saprophytic; 6, autotrophic
- Relation to free oxygen: 1, strict aerobe; 2, facultative anaerobe; 3, strict anaerobe; 4, microaerophile
- In nitrate media: 0, neither nitrite nor gas; 1, both nitrite and gas; 2, nitrite but no gas; 3, gas but no nitrite
- Chromogenesis: 0, none; 1, pink; 2, violet; 3, blue; 4, green; 5, yellow; 6, orange; 7, red; 8, brown; 9, black
- Other photic characters: 0, none; 1, photogenic; 2, fluorescent; 3, iridescent
- Indole: 0, negative; 1, positive
- Hydrogen sulfide: 0, negative; 1, positive
- Hemolysis: 0, negative; 1, positive
- Methemoglobin: 0, negative; 1, positive

PROTEIN LIQUEFACTION OR DIGESTION
- Gelatin: 0, negative; 1, positive
- Casein: 0, negative; 1, positive
- Egg albumin: 0, negative; 1, positive
- Blood serum: 0, negative; 1, positive

INDICATOR REDUCTION
- Litmus: 0, negative; 1, positive
- Methylene blue: 0, negative; 1, positive
- Janus green: 0, negative; 1, positive

Rennet production: 0, negative; 1, positive

SUPPLEMENTARY DATA

TEMPERATURE RELATIONS

Medium.....pH.....
Optimum temperature for growth.....°C.
Maximum temperature for growth.....°C.
Minimum temperature for growth.....°C.
THERMAL DEATH POINT: Time 10 minutes:.....°C.
Medium.....pH.....

THERMAL DEATH TIME:
Medium.....pH.....

Temp.	Time	Temp.	Time
.....°C.	.....min.	.....°C.	.....min.
.....°C.	.....min.	.....°C.	.....min.
.....°C.	.....min.	.....°C.	.....min.
.....°C.	.....min.	.....°C.	.....min.
.....°C.	.....min.	.....°C.	.....min.

CHROMOGENESIS

Gelatin.....
Agar.....
Potato.....
.....

OTHER PHOTIC CHARACTERS

Photogenesis on.....
Iridescence on.....
Fluorescence in.....
.....

RELATION TO REACTION (pH) OF MEDIUM

Medium.....
Optimum for growth: *about pH*.....
Limits for growth: *from pH*.....to.....

RELATION TO FREE OXYGEN

Method.....
Medium.....Temp.....°C.
Aerobic growth: *absent, present, better than anaerobic growth, micro-aerophilic*
Anaerobic growth: *absent, occurs in presence of glucose, of sucrose, of lactose, of nitrate; better than aerobic growth*
Additional data:.....
.....
.....

MILK

Temperature.....°C.

Reaction:	.....d. .....;	.....d. .....;	.....d. .....
Acid curd:	.....d. .....;	.....d. .....;	.....d. .....
Rennet curd:	.....d. .....;	.....d. .....;	.....d. .....
Peptonization:	.....d. .....;	.....d. .....;	.....d. .....

LITMUS MILK

Temperature.....°C.

Reaction:	.....d. .....;	.....d. .....;	.....d. .....
Acid curd:	.....d. .....;	.....d. .....;	.....d. .....
Rennet curd:	.....d. .....;	.....d. .....;	.....d. .....
Peptonization:	.....d. .....;	.....d. .....;	.....d. .....

Reduction of litmus *begins in*.....*days, ends in*.....*days*

ACTION ON ERYTHROCYTES

Cells:.....
Method: *plate, broth, filtrate*
Hemolysis: *negative, positive*
Methemoglobin: *negative, positive*

PRODUCTION OF INDOLE

Medium.....
Test used.....
Indole *absent, present in*.....*days*

PRODUCTION OF HYDROGEN SULFIDE

Medium.....
Test used.....
H_2S *absent, present in*.....*days*

ACTION ON NITRATES

Medium.....Temp.....°C.

Nitrite:	.....d. .....;	.....d. .....;	.....d. .....; .....d. .....
Gas (N_2):	.....d. .....;	.....d. .....;	.....d. .....; .....d. .....

Medium.....Temp.....°C.

Nitrite:	.....d. .....;	.....d. .....;	.....d. .....; .....d. .....
Gas (N_2):	.....d. .....;	.....d. .....;	.....d. .....; .....d. .....

Ammonia production (in amino-N-free nitrate medium): *negative, positive*
Complete disappearance of nitrate in.....medium: *negative, positive*
Disappearance of 2 p.p.m. nitrite in.....medium: *negative, positive*

REDUCTION OF INDICATORS

Medium.....pH.....Temp.....°C.

Indicator	Conc.	Reduction:	
.....	.....%	.....hr. .....;	.....hr.
.....	.....%	.....hr. .....;	.....hr.
.....	.....%	.....hr. .....;	.....hr.
.....	.....%	.....hr. .....;	.....hr.

STAINING REACTIONS

Gram: .....d. .....; .....d. .....; .....d. .....; .....d. .....
Method.....
Spores: Method.....
Capsules: Method.....
Medium.....
Flagella: Metbod.....
Special Stains:.....
.....
.....

ADDITIONAL TESTS

Methyl red: *negative, positive*
Voges-Proskauer: *negative, positive*
Growth in sodium citrate: *absent, present*
Growth in uric acid: *absent, present*
Hydrolysis of starch: *complete (iodine colorless); partial (iodine reddish-brown); none (iodine blue)*
Nitrogen obtained from the following compounds:.....
.....
.....
.....

PATHOLOGY

ANIMAL INOCULATION

Medium used.....Age of culture.....Amount.....Incubation period.....

		Whole culture			Cells			Filtrate		
Animal										
Type of Injection	Subcutaneous	*								
	Intraperitoneal									
	Intravenous									
	Per os									

*In each instance where pathogenicity is observed, indicate location of lesion, and type, e. g. edema, histolysis, gas, hemorrhage, ulcer, diphtheritic, etc.

ANTIGENIC ACTION

Animal.....Medium used.....Age of culture.....
Type injection.....Number of injections.....
Culture causes production of *cytolysins, agglutinins, precipitins, antitoxin.*
Specificity: Antibodies produced effective against other antigens as follows.....
.....
Immune sera from.....
.....effective against this organism as antigen

SPECIAL TESTS

This DESCRIPTIVE CHART presented at the annual meeting of the SOCIETY OF AMERICAN BACTERIOLOGISTS, Dec. 28, 1934, by the Committee on Bacteriological Technic.
Prepared by a sub-committee consisting of M. W. Jennison and H. J. Conn.

Name of organism ..... Studied by ..... Culture No. .....

Source ..... Habitat ..... Date .....

Descriptions (*Underscore required terms.*)	Sketches
CELL MORPHOLOGY Medium:	Temp. °C.
Vegetative cells: Age: Form and arrangement: *streptococci, diplococci, micrococci, sarcinae, rods, commas, spirals, branched rods, filaments.* Motility in broth: Flagella: Size: Irregular forms: Sporangia: *none, rods, spindles, elliptical, clavate, drumstick.* Age: Endospores: Shape: *spherical, ellipsoid, cylindrical.* Position: *central to excentric, terminal, subterminal.*	
STAINING CHARACTERISTICS Gram: Age: Method: Special stains:	
AGAR STROKE Age:	Temp. °C.
Amount of growth: *scanty, moderate, abundant.* Form: *filiform, echinulate, beaded, spreading, rhizoid.* Consistency: *butyrous, viscid, membranous, brittle.* Chromogenesis: ; *fluorescent, iridescent, photogenic.*	
AGAR COLONIES Age:	Temp. °C.
Form: *punctiform, circular, filamentous, rhizoid, irregular.* Elevation: *effuse, flat, raised, convex.* Surface: *smooth, contoured, radiate, concentric, rugose.* Margin: *entire, undulate, erose, filamentous, curled.* Density: *opaque, translucent.*	
NUTRIENT BROTH Age:	Temp. °C.
Surface growth: *none, ring, pellicle, flocculent, membranous.* Subsurface growth: *none, turbid, granular.* Amount of growth: *scanty, moderate, abundant.* Sediment: *none, granular, flocculent, viscid, flaky.*	
GELATIN STAB Age:	Temp. °C.
Liquefaction: *none, crateriform, infundibuliform, napiform, saccate, stratiform.* Rate: *slow, moderate, rapid.*	
OTHER MEDIA Age:	Temp. °C.

FERMENTATION		Temp. °C.				
Medium: Carbohydrate: % Indicator:		Glucose	Lactose	Sucrose		
Acid in	days					
Acid in	days					
Gas in	days					
Gas in	days					

ACTION ON MILK	Temp. °C.					
Indicator:	Days					
Reaction						
Acid curd						
Rennet curd						
Peptonization						
Reduction (before coagulation)						

DESCRIPTIVE CHART FOR INSTRUCTION

ACTION ON NITRATES

Medium: Temp. °C.

Nitrite: .....d. ; .....d. ; .....d.

Gas (N): .....d. ; .....d. ; .....d.

HYDROGEN SULFIDE PRODUCTION

Medium: Age:

H_2S: *present, absent.* Temp. °C.

TEMPERATURE RELATIONS

Growth in refrigerator (°C.): *present, absent.*

Growth at room temperature (°C.): *present, absent.*

Growth at 37° C.: *present, absent.*

Growth at 50° C.: *present, absent.*

INDOLE PRODUCTION

Medium: Age:

Method: Temp. °C.

Indole: *present, absent.*

RELATION TO FREE OXYGEN

Medium: Age:

Method: Temp. °C.

Aerobic growth: *absent, present, better than anaerobic growth, poorer than anaerobic growth.*

Anaerobic growth: *present, absent.*

ADDITIONAL TESTS

This simplified Descriptive Chart recommended for instruction purposes by the Committee on Bacteriological Technic of the Society of American Bacteriologists and put on sale provisionally during 1939.

amount of solution No. 1. Replace the plug and push down until an inch or an inch and a half above the surface of the culture. Place the tube upright in a boiling water-bath and heat for 15 minutes without letting the culture solution come in contact with the plug. The appearance of a red color on the plug indicates the presence of indole.

The Kováes (1928) test is a simplification of that of Böhme, using only one solution; it is now the method of choice in many laboratories:

Para-dimethyl-amino-benzaldehyde	5 g.
Amyl or butyl alcohol	75 ml.
Hydrochloric acid, concentrated	25 ml.

This reagent may be used as in the Böhme test, but no solution 2 is required.

The Gnezda (1899) oxalic acid test is made as follows: dip a strip of filter paper in a warm saturated solution of oxalic acid; on cooling, this is covered with crystals of the acid. Dry the strip of paper thoroughly (sterilization by heat seems unnecessary), and insert into the culture tube under aseptic conditions, bent at such an angle that it presses against the side of the tube and remains near the mouth. Reinsert the plug and incubate the culture. If indole is formed, the oxalic acid crystals take on a pink color.

It is recommended that the Goré or the Kováes test be used in a routine way. In interpreting the results obtained it must be remembered that when the reagents are added directly to the medium they react with alpha-methyl-indole as well as with indole itself; but as the former compound is non-volatile it cannot react to the Goré or Gnezda tests. Hence the Ehrlich test unmodified is less specific for indole than the Goré modification or the Gnezda test.

Some samples of para-dimethyl-amino-benzaldehyde and of amyl and butyl alcohol have been found unsatisfactory for the indole test. It is well, therefore, to check new supplies of these chemicals against samples known to be satisfactory.

In early editions of this section of the Manual, the vanillin, or Steensma, test was also described. It is now omitted, as it is regarded as unreliable; Fellers and Clough (1925), for instance, have shown it to give too high a percentage of positive reactions unconfirmable by any other test.

The Production of Hydrogen Sulfide

Hydrogen sulfide is generally detected in bacterial cultures by observing the blackening which it produces in the presence of salts of certain metals, such as lead, iron or bismuth, due to the dark color of the sulfide of these metals. Two methods have been utilized for employing these tests: one by incorporating the metallic salt in the medium, and the other by using a test strip of filter paper impregnated with the metallic salt in question.

In early editions of this Manual four media containing either lead or iron salts were given. The lead salt media, however, were discredited

some time ago because of the toxic properties of these salts; and Hunter and Crecelius (1938) show the superiority of bismuth media over iron media. ZoBell and Feltham (1934), moreover, have shown distinct advantages from the use of lead acetate test strips, without any of these metallic salts in the media. The advantage of the test strip technic is that it is more sensitive and does not introduce the possibility of inhibiting the bacterial growth if the concentration of metallic salt in the medium is too great. It is important, as emphasized by Hunter and Crecelius, that the indicator and method employed be stated when results are given. Untermohlen and Georgi (1940) suggest use of nickel or cobalt salts, but specially emphasize the variations in results with different media and indicators.

When using the test strip technic the bacteria may be grown in ordinary broth, peptone solution alone, or a peptone agar suitable to the organism in question. One must be certain that the peptone contains available sulphur compounds. This can be determined by running a check tube inoculated with a slow hydrogen sulfide producer. For this procedure the test strip should be prepared by cutting white filter paper into strips approximately 5 x 50 mm., soaking them in a saturated solution of lead acetate, sterilizing them in plugged test tubes and drying in an oven at 120°C. One of these strips should be placed in the mouth of the culture tube before incubation in such a position that one-quarter to one-half of the strip projects below the cotton plug. These tubes should be incubated at about the optimum temperature of the organism under investigation and examined daily to notice whether or not blackening of the test strip has occurred.

Because of the inconvenience of the test strip technic, media in which iron salts are incorporated are now generally preferred. A dehydrated medium of such composition is available and has been found quite satisfactory.

Quantitative methods for determining hydrogen sulfide production are given in Leaflet VI.

Liquefaction of Gelatin

The conventional method of determining liquefaction, which has been given with but slight modification in all the reports on methods is as follows:

Make a gelatin stab (plain 12% gelatin) and incubate 6 weeks at 20°C., provided the organism under investigation will grow at that temperature. Care must be taken to observe whether the organisms produce rapid and progressive liquefaction or merely slow liquefaction not extending far from the point of inoculation. In the latter case the liquefaction may be due merely to endo-enzymes that are released from the cell after death and may not be what is generally called "true liquefaction" (that is, the process resulting from the

action of enzymes diffusing out of actively growing cells). Some slow liquefiers are true liquefiers, however; and the distinction between slow and rapid liquefaction must be regarded as very artificial.

In early editions of this Leaflet the Frazier (1926) method was given, but it was omitted from later editions as not proving practicable. A recent modification of it by Smith (1946), however, proves useful, and has two advantages over the gelatin stab method: (1) it does not require low temperature incubation; (2) it is more sensitive in the case of weak liquefiers. The procedure is as follows: Streak culture on a plate of nutrient agar containing 0.4% of gelatin. Incubate at 28°C for 2–14 days according to rate of growth. Cover plate with 8–10 ml. of a solution of 15 g. of $HgCl_2$ in 100 ml. distilled water and 20 ml. concentrated HCl. This reagent forms a white opaque precipitate with the unchanged gelatin, but a liquefier is surrounded by a clear zone.

There is another method recommended for organisms that do not grow at 20°C. By this technic an inoculated tube of gelatin is incubated at 37°C., or whatever temperature may be the optimum, and then after incubation the tubes are placed in a cold water bath or in a refrigerator to determine whether or not the gelatin is still capable of solidifying. Suitable uninoculated controls must always be run in parallel, especially if the optimum growth conditions for the organism necessitate prolonged exposure of the gelatin to hydrolysis by mild acid, alkali or heat. In addition, precautions should always be taken to prevent evaporation of moisture which might conceivably tend to obscure a slow liquefaction. This method has the advantage of rarely giving positive results except in case of "true liquefaction". On the other hand, it may well fail to detect cases of real liquefaction that have proceeded so slowly that the gelatin can still set even after several weeks's incubation. The significance of this test can be increased by using weaker than normal gelatin,—4% gelatin, for example, or even less.

Other methods designed to give more technical information on the subject are given in Leaflet VI.

Cleavage of Sugars, Alcohols, and Glucosides

Fermentable substance to employ. Quite a wide range of pure alcohols and carbohydrates is available for use in fermentation tests. In routine work the choice is often limited to the more common and less expensive substances; but in special research work economy is of less importance. The three sugars, glucose, sucrose, and lactose, and the alcohols, glycerol and mannitol, are most widely employed because they are readily available. Whether these compounds give valuable information depends upon the group of organisms being studied. If the group, like the colon group, is capable of fermenting nearly all these substances, these readily fermented sugars and alcohols may have very little value in separating the species one from another; one must then employ one or more of the rarer compounds In other words the selection is based upon the group of bacteria under investigation.

The list of fermentable substances often used in such work is given in Leaflet VI.

Basal Medium. The compound to be tested must be added to some basal medium suited to the group of organisms under investigation. For routine work it is best to employ two such basal media; namely, beef extract peptone broth and beef extract peptone agar, selecting one or the other according to whether the organisms under investigation grow better in liquid or solid media. These media should be prepared as directed in Leaflet II. It should be noted that some commercial peptones contain fermentable sugars (Vera, 1949); hence care must be exercised in regard to the peptone selected, and controls must be run.

Another important basal medium is the synthetic formula (Ayers, Rupp and Johnson) given on p. II_{44}–14 of Leaflet II. This can be used only for organisms that utilize ammonium salts as a source of nitrogen; but is valuable for organisms that cause misleading changes in reaction from proteins or which produce so little acid that it does not become evident in a highly buffered medium.

One should notice particularly whether or not good growth is obtained in any or all of these media after adding the fermentable substance under investigation. If poor growth or none is obtained in the broth and on the agar, one should vary the basal medium employed, following the suggestions given in Leaflet VI.

If a culture is to be studied in liquid, the media should be sterilized in fermentation tubes; if on solid media, agar slants should be used—see Conn and Hucker (1920). Agar slants may be inoculated either on the surface alone or partly on the surface and partly in a stab at the base. It has been found in practice that if much gas is produced it may occur at the very base of the column of agar even when all the growth seems to occur on the surface; but if there is reason to suspect that gas production is being overlooked, shake cultures may be used in addition to the agar slant.

Demonstration of Cleavage. Utilization of the sugar (or other fermentable substance) may be indicated by a chemical determination showing its partial or complete disappearance, or by the demonstration of the end-products of fermentation. These end-products are generally organic acids, sometimes accompanied with the evolution of gases, e. g., free hydrogen, carbon dioxide, or occasionally methane. Determinations of the amount of sugar remaining or of the nature of the organic acids produced are very valuable in discriminating investigations, but require time-consuming chemical work that is difficult to employ in the routine examination of large numbers of cultures.

These chemical methods are referred to in more detail elsewhere (Leaflet VI). In many instances, however, a sufficient amount of information is obtained merely by demonstrating an increase in acid or the presence of gas.

For routine work in the case of organisms concerning which little advance information is at hand, the use of indicators is especially valuable in determining whether or not production of acid has occurred. It must be remembered, however, that in many instances more useful and significant information can be obtained by means of titration. (See Leaflet VI.)

When the indicator method is employed, the indicators may be incorporated with the media in the first place or may be added subsequently when the final reaction is being determined. If they are added when determining final reaction, the color obtained should be compared with color standards (see Leaflet IX) in order to secure accuracy. The use of indicator media is less accurate, but is a much more rapid procedure; when the cultures are growing on agar, moreover, it is the only satisfactory procedure.

When using indicator media, make them up according to the directions given on pp. II_{44}-7, 8, of Leaflet II The indicator most commonly added is brom cresol purple; but with organisms producing considerable acid, brom cresol green or even brom phenol blue may be employed. When studying a series of unknown organisms it is often advisable to inoculate all onto the prescribed sugar medium with brom cresol purple; later those that show acid may be reinoculated onto the same medium with brom cresol green; and subsequently those positive to this indicator upon the same medium with brom phenol blue. If it is decided to observe the production of alkalinity as well as acidity, one may employ brom thymol blue or better a mixture of brom cresol purple with cresol red, making up the medium as directed on p. II_{44}-8 of Leaflet II; in a solid medium this practice is often of value as it may show the production of acid in one part of the tube, and of alkalinity in another.

TABLE 1

THE SENSITIVE RANGES OF THE THREE INDICATORS RECOMMENDED FOR USE IN INDICATOR MEDIA

pH:	7.0	6.0	5.5	5.0	4.0	3.0
Br. Cres Purple:	Purple | ≺Sensitive range≻			|Yellow.....		
Br Cres. Green:	.....Blue | ≺Sensitive range≻				|Yellow	
Br. Phenol Blue:	.....Blue | ≺Sensitive range≻					|Yellow

With indicator media it is difficult to learn the exact reaction by reference to color standards, but a good estimate as to hydrogen-ion concentration can be obtained by inspection, particularly when three tubes are used, one with each of the three indicators recommended above. For this purpose Table 1, showing the relation of the ranges of these three indicators to each other, will be found useful.

After some experience a bacteriologist can usually devise some method for recording on the Chart, by a system of numerals or + signs, the strength of reaction observed with each indicator employed; such a system often proves practical for comparative purposes, but gives no very definite information as to final H-ion concentration.

FIG. 1. THE SMITH FERMENTATION TUBE

Gas production in liquid media is ordinarily measured in percentage of gas in the closed arm of the Smith or the Durham fermentation tube. The Durham tube consists of small test tube (e. g. 75 x 10 mm.) inverted in a large tube (e. g. 150 x 18 mm.). In the case of solid media it is recorded as present or absent according to whether or not bubbles or cracks are present in the agar. This test is especially valuable if the organism is tested in a shake culture; but the presence of gas can usually be detected in an ordinary agar slant. These tests for gas production are chiefly useful if the organism produces primarily hydrogen; if the gas is all carbon dioxide little or none will accumulate in the fermentation tube because of the great solubility and rapid diffusion into the air. A convenient, simple method that has been proposed for the accurate determination of carbon dioxide is that of Eldredge and Rogers (1914). (See Leaflet VI.)

Interpretation of Results. In case an organism produces gas or considerable increase in acidity in either broth or beef extract peptone agar in the presence of some fermentable substance, and this does not occur in the basal medium without the addition of the fermentable substance, it may safely be concluded that cleavage of this substance has occurred. Very often for routine diagnostic purposes such information is enough. To understand the true action of the organism on any carbon compound, however, much more investigation must be made as explained elsewhere. (See Leaflet VI). This is particularly necessary in the case of organisms that produce a small amount of acid in some tubes but not in others containing the same carbon source, and in cases where the addition of some carbon source results in a distinctly improved growth without the appearance of demon-

strable acid or gas. In routine work, accordingly, one should record as positive only those organisms that produce considerable acid or gas from a given compound and as negative only those that consistently fail to show any acid or gas, nor any increase of growth when supplied with the carbon compound under investigation. All others should be regarded as border-line cultures, calling for further investigation as given in Leaflet VI.

Hydrolysis of Starch

The breaking down of starch is rather more complicated than that of sugars because of the extensive hydrolysis that is necessary before it can be utilized by the bacteria. The first stage of this process is generally known as diastatic action because of the similarity to that brought about by the enzyme diastase. The final end result is usually an increase in acid, so one may obtain good evidence as to the utilization of starch by substituting it for sugar in the above methods (pp. v_{49}15–17) and determining acid produced or increase in H-ion concentration. It is often desirable, however, to secure evidence as to the intermediate products and as to whether the starch has been entirely consumed or not; and various methods have been proposed for this purpose.

This test may be made on raw starch, dissolved by boiling, or on the so-called "soluble starch." The latter is a partly hydrolyzed product; but it is often used as "starch" in this test because its iodine reaction is like that of true starch and different from that given by typical dextrins. If soluble starch is used, its true nature must be taken into account; but at the same time it must be remembered that true starch is partly hydrolyzed when sterilized in culture media, and even cultures growing in such a substratum are not furnished with raw starch as the sole carbohydrate. When such media are filtered, possibly "soluble starch" is all that remains.

A satisfactory method has been proposed by Eckford (1927) for learning the type of action on starch brought about by organisms capable of making good growth in broth. The same method may be adapted to organisms which prefer some other liquid medium by substituting it for broth in Eckford's method. The procedure, however, is not well adapted to those bacteria that fail to grow well in liquid medium. The technic is as follows:

Add 0.2% soluble starch to broth and incubate cultures a week to ten days. Examine on 2nd, 4th, 7th and 10th days for hydrolysis of starch, production of acid, and reduction of Fehling's solution. For this test a drop is placed in a depression on a porcelain plate and a larger quantity in a serological test tube. The latter is tested for acid production with an indicator of the proper pH-range. To the drop on the

plate add a drop of dilute iodine solution and read reaction as follows: if blue, no hydrolysis; if reddish brown, partial hydrolysis with production of erythrodextrin; if clear, hydrolysis complete, with production of dextrin or perhaps glucose. The tubes showing complete hydrolysis may be tested for reducing sugar with Fehling's solution.

For bacteria that do not grow well in liquid media, no better method has yet been proposed than the plate technic given in all previous editions of the Manual with little modification. This method has its disadvantages, but is often useful; it is as follows:

Use beef-extract agar containing 0.2% of soluble starch. Pour it into a Petri dish, and after hardening make a streak inoculation on its surface. Incubate at optimum temperature for the organism under investigation. Observations are to be made on the second day for rapidly growing organisms but not until the 7th day for the more slowly growing ones. To make the test, flood the surface of the Petri dishes with Lugol's iodine or with a saturated solution of iodine in 50% alcohol. The breadth of the clear zone outside of the area of growth indicates the extent of starch destruction. By means of a simultaneous inoculation on another plate containing the same medium with brom cresol purple as an indicator one may at the same time learn whether or not acid is produced as an end-product.

THE METHYL RED AND VOGES-PROSKAUER TESTS

Special tests as to cleavage of glucose are commonly made in the differentiation of the organisms of the colon-aerogenes group. The medium ordinarily employed is as follows: 5 g. proteose peptone (Difco, Witte's, or some brand recognized as equivalent), 5 g. C. P. glucose, 5 g. K_2HPO_4 in 1000 ml. distilled water. The dry potassium phosphate should be tested before using in dilute solution to see that it gives a distinct pink color with phenolphthalein. According to Smith (1940), the K_2HPO_4 in this medium should be replaced with the same amount of NaCl, if the tests are to be carried out on aerobic spore-formers. Tubes should be filled with 5 ml. each and each culture should be inoculated into duplicate (or triplicate) tubes for each of the two tests. Incubation should be at optimum temperature of the organism under investigation, and tubes should be incubated 2–7 days, according to the rate of growth of the organism in question. Although the same medium is used for both the methyl red and the Voges-Proskauer tests, they must be performed in separate tubes. The latter test depends upon the production of acetyl-methyl-carbinol from the glucose; see Leaflet VI.

A positive methyl red reaction is regarded as being present when the culture is sufficiently acid to turn the methyl red (0.1 g. dissolved in 300 ml. 95% ethyl alcohol and diluted to 500 ml. with distilled water) a distinct red; a yellow color with the methyl red indicator is regarded as a negative reaction, while intermediate shades should be considered doubtful.

For the Voges-Proskauer reaction, according to the "Standard Methods" of the A. P. H. A. (1946), to 1 ml. of culture add 0.6 ml. of 5% α-naphthol in absolute alcohol and 0.2 ml. of 40% KOH. The development of a crimson to ruby color in the mixture from 2 to 4 hours after adding the reagents constitutes a positive test for acetyl-methyl-carbinol. Results should be read not later than 4 hours after addition of the reagents.

Various other tests have been suggested for this reaction, both to obtain results more quickly and because some organisms apparently give different results with different tests. In any case, weakly positive reactions may be obscured by the color of the reagent. A procedure which has given excellent results with many thousand cultures run by a member of the committee (C.A.S.) is the creatine test of O'Meara, as modified by Levine, Epstein and Vaughn (1934). In this procedure the test reagent added to the culture is 0.3% creatine in 40% KOH. This reagent deteriorates rapidly at temperatures over 50°C. but may be kept 2 weeks at room temperature (22–25°C.) or for 4 to 6 weeks in a refrigerator.

A recent modification of Coblentz (1943) is similar to the A. P. H. A. method, but uses a massive inoculum in broth from an infusion-agar slant culture, followed by incubation of the broth for 6 hours. Also, the 40% KOH has 0.3% creatine added to it to intensify the reaction. After addition of the reagents (α-naphthol and KOH-creatine) the culture is shaken vigorously for one minute; a positive reaction is characterized by an intense rose-pink color developing in a few seconds to ten minutes.

A more detailed and accurate procedure for determining acetyl-methyl-carbinol is given in Leaflet VI.

Acid Production in Milk

Acid production in milk may be determined very simply; but the opacity of the milk must be taken into account if accurate determinations are desired. The milk must be considerably diluted before adding indicator for comparison with a buffer standard.

Indicator milk is often useful. Litmus has been used most frequently, as it indicates reduction as well as pH changes (although roughly). Neutral litmus milk (about pH 6.8) has a lavender color, which becomes red with acid production or blue with production of alkalinity. Reduction is indicated by a partial or complete fading of the color. The use of litmus milk has been seriously criticized because of the inaccurate nature of litmus as a pH indicator; nevertheless the differences it brings out have enough practical value so that it has not yet been superceded by any other indicator in milk.

The use of brom cresol purple, as was recommended by Clark and Lubs (1917) does not show changes in O–R potential.

TABLE 2

DEGREES OF ACIDITY EASILY RECOGNIZED IN MILK

ACIDITY	INDICATOR, REACTION, ETC.	APPROXIMATE pH-VALUE
"Neutral".....	Same color with brom cresol purple as sterile milk—i. e. blue to gray-green	6.2–6.8
"Weak".....	Color with brom cresol purple lighter than in sterile milk—i. e. gray-green to greenish yellow	5.2–6.0
"Moderate".....	Yellow with brom cresol purple. Not curdled	4.7–6.0
"Strong".....	Curdled. Blue or green to brom phenol blue	3.4–4.6
"Very strong"..	Yellow to brom phenol blue	Under 3.4

During the second World War, stimulated by the unavailability of litmus, Ulrich (1944) proposed using instead of litmus a mixture of methylene blue and chlor phenol red. This combination added to milk shows, for many species, all that litmus does and in addition shows a distinction between acid reduction and alkaline reduction; but in using it one must accustom himself to alkalinity being indicated by red, acid by yellow or green. When using litmus or the Ulrich combination, one must distinguish between reduction before and after coagulation, as the latter is often of little significance.

It is possible to recognize the five degrees of acidity listed in Table 2 by the use of brom cresol purple (either in the milk before inoculation or added after incubation), the subsequent addition of brom phenol blue, and observation as to the presence of curdling. This is only a rough method of measurement; but in the routine study of milk cultures it will often be found valuable.

H. C. Brown (1922) proposed condensed milk diluted with 4 parts water containing phenol red. The reaction is adjusted by addition of alkali until first appearance of a brick red. Subsequent changes of reaction in either direction can be observed.

Rennet Production

The production of the enzyme, rennet (lab), can sometimes be recognized in litmus milk by noticing the occurrence of coagulation without the appearance of acid. It is often obscured by simultaneous digestion, however, and two other methods have been proposed which often show rennet production with cultures that fail to show it when inoculated directly into milk.

Conn (1922) grows bacteria in milk sterilized in the usual manner; after the appearance of whey or peptonized milk, 0.5 ml. is transferred to 10 ml. of unsterilized milk and placed in a 37° incubator. Examinations are made every 5 minutes for the first half hour, and at less frequent periods thereafter for a few hours longer. First appearance of coagulation is noted.

Gorini (1932) obtains vigorous growth on an agar slant, then covers the growth with

milk, fractionally sterilized at temperatures not over 100° so as not to alter the color of the milk. The growth is mixed with the milk by use of a platinum needle, and the tube is incubated at 37° until coagulation occurs.

Although the Committee is not prepared to recommend either method, it is felt that by a combination of the two a good indication of rennet production can be obtained.

REFERENCES

Amer. Public Health Assn. 1946. Standard Methods for the Examination of Water and Sewage. *9th Ed.* Published by the Association, New York.

Bartholomew, J. W. and Umbreit, W. W. 1944. Ribonucleic acid and the Gram stain. J. Bact., **48**, 567–78.

Böhme, A. 1905. Die Anwendung der Ehrlichschen Indolreaktion für bacteriologische Zwecke. Centbl. f. Bakt., I Abt. Orig., **40**, 129–133.

Brown, H. C. 1922. Use of phenol red as an indicator for milk and sugar media. Lancet, **202**, 842.

Clark, W. M., and Lubs, H. A. 1917. A substitute for litmus for use in milk cultures. J. Agric. Research, **10**, 105–111

Coblentz, J. M. 1943. A rapid test for acetyl methyl carbinol production. Amer. J. Pub. Hlth., **33**, 815.

Committee on Bact. Technic. 1927. Variability of the Gram reaction. Stain Technology, **2**, 80–87.

Conn, H. J. 1922. A method of detecting rennet production by bacteria. J. Bact., **7**, 447–8.

Conn, H. J., and Hucker, G. J. 1920. The use of agar slants in detecting fermentation. J. Bact., **5**, 433–435.

Conn, H. J., and Wolfe, Gladys E. 1938. Flagella staining as a routine test for bacteria. J. Bact., **36**, 517–20.

Eckford, Martha O. 1927. Thermophilic bacteria in milk. Amer. J. of Hyg., **7**, 201–221. (See p. 208.)

Eldredge, E. E., and Rogers, L. A. 1914. The bacteriology of cheese of the Emmental type. Centbl. f. Bakt., II Abt., **40**, 5–21, (See p. 13.)

Fellers, C. R., and Clough, R. W. 1925. Indol and skatol determination in bacterial cultures. J. Bact., **10**, 105–133.

Fisher, P. J. and Conn, Jean E. 1942. A flagella staining technic for soil bacteria. Stain Technology, **17**, 117–121.

Foth, 1892. Zur Frage der Sporenfärbung. Centbl. f. Bakt. **11**, 272–278.

Frazier, W. C. 1926. A method for the detection of changes in gelatin due to bacteria. J. Inf. Dis., **39**, 302–9.

Frieber, W. 1921. Beitrage zur Frage der Indolbildung und der Indolreacktionen sowie zur Kenntnis des Verhaltens indolnegativer Bacterien. Centbl. f. Bakt., I Abt. Orig., **87**, 254–277.

Gnezda, J. 1899. Sur les reactions nouvelles des bases indoliques et des corps albuminoides. Com. Rend., Acad. Sci., **128**, 1584.

Goré, S. N. 1921. The cotton-wool plug test for indole. Indian J. of Med. Res., **8**, 505–507.

Gorini, C. 1932. The coagulation of milk by *B. typhosus* and other bacteria considered inactive on milk. J. Path. and Bact., **35**, 637.

Henry, H. and Stacey, M. 1943. Histochemistry of the Gram-staining reaction for microorganisms. Nature, **151**, 671.

HOLMAN, W. L., and GONZALES, F. L. 1923. A test for indol based on the oxalic acid reaction of Gnezda. J. Bact., **8**, 577–583.

HUNTER, C. A., and CRECELIUS, H. G. 1938. Hydrogen sulphide studies. I Detection of hydrogen sulphide in cultures. J. Bact. **35**, 185–196.

KOSER, S. A., and GALT, R. H. 1926. The oxalic acid test for indol. J. Bact., **11**, 293–303.

KOVÁCS, N. 1928. Eine vereinfachte Methode zum Nachweis der Indolbildung durch Bakterien. Zts. f. Immunitats. **55**, 311–15.

LEVINE, MAX, EPSTEIN, S. S. and VAUGHN, R. H. 1934. Differential reactions in the colon group of bacteria. Amer. J. Pub. Hlth. 24, 505–10.

MAGOON, C. A. 1926. Studies upon bacterial spores. J. Bact., **11**, 253–83. (See p. 261–4.)

MOELLER, H. 1891. Über eine neue Methode der Sporenfärbung. Centbl. f. Bakt. **10**, 273–277.

SMITH, N. R. 1940. Factors influencing the production of acetyl-methyl-carbinol by the aerobic spore-formers. J. Bact. **39**, 575.

SMITH, N. R. 1946. Aerobic mesophilic sporeforming bacteria. U. S. Dept. of Agric., Misc. Publ. No. 559.

TITTSLER, R. P. 1930. The reduction of nitrates to nitrites by *Salmonella pullorum* and *Salmonella gallinarum*, J. Bact., **19**, 261–267.

TITTSLER, R. P., and SANDHOLZER, L. A. 1936. The use of semi-solid agar for the detection of bacterial motility. J. Bact. **31**, 575–80.

ULRICH, J. A. 1944. New indicators to replace litmus in milk. Sci., **99**, 352.

UNTERMOHLEN, W. P. and GEORGI, C. E. 1940 A comparison of cobalt and nickel salts with other agents for the detection of hydrogen sulfide in bacterial cultures. J. Bact. **40**, 449–59.

VERA, H. D. 1949. Accuracy and sensitivity of fermentation tests. Abs. of Papers, Soc. Amer. Bact., 49th Gen. Meeting, p. 6.

WALLACE, G. I., and NEAVE, S. L. 1927. The nitrite test as applied to bacterial cultures. J. Bact., **14**, 377–384.

ZIPFEL, H. 1912. Zur Kenntnis der Indolreaktion. Centbl. f. Bakt., I Abt. Orig., **64**, 65–80.

ZO BELL, C. A., and FELTHAM, CATHERINE B. 1934. A comparison of lead, bismuth, and iron as detectors of hydrogen sulphide produced by bacteria. J. Bact. 28, 169–178

LEAFLET VI

FURTHER BIOCHEMICAL METHODS

9th EDITION
Pure Culture Study of Bacteria. Vol. 10, No. 4
NOVEMBER, 1942

Revised by
C. H. WERKMAN

Committee members assisting in the revision:
Barnett Cohen, W. W. Jennison and J. A. Kennedy

LEAFLET VI

FURTHER BIOCHEMICAL METHODS

Introduction

Leaflet V dealing with routine tests for the Descriptive Chart describes certain of the simpler biochemical tests used quite generally in the study of bacteria. There are, however, a considerable number of biochemical tests which are in fairly common use in the pure culture study of bacteria but which are not included in Leaflet V for one or the other of two reasons: they either apply only to certain special groups of bacteria or they involve such intensive chemical study that they cannot easily be used in routine work. The methods given in this Leaflet, therefore, are to be used primarily in the study of special groups of bacteria after a preliminary survey has established most of their general morphological and physiological characteristics. In such cases it is very often desired to make a more careful physiological study of a few strains, and the routine tests given in Leaflet V or in previous editions of Leaflet VI are entirely inadequate for any detailed biochemical investigations.

The sixth (1935) edition of Leaflet VI was the first to deal with any but routine biochemical tests, and accordingly its title was then changed to show the new field covered by it. The first editions of the Leaflet under its new title are necessarily incomplete. The object of this Manual has always been to list methods that have actually been used by members of the Committee and have been found practical in pure culture study of bacteria. Inasmuch as the new field now covered by Leaflet VI is a very broad one, the present Committee members have not had experience with procedures in all the lines that should be included. Accordingly, it is planned to make the first editions quite brief, with the intention of revising this text and adding new material with each successive edition until the field is more adequately covered. *Assistance from users will be greatly appreciated in making suggestions as to what should be covered in future editions.* It is hoped that the present edition will be of value in pointing the way to methods for those who are confused by the multiplicity of procedures in the literature.

In making a physiological study of any kind of bacteria, special consideration should be given to the question of variation as discussed at the beginning of Leaflet V. Strain variations, in fact, are more likely to affect biochemical reactions than matters of morphology. It is, accordingly, important that no conclusions be based upon single determinations, nor even upon several determinations when all are made upon a single strain. It cannot be overemphasized that a physiological study of any type of bacteria should always be based upon repeated

determinations with several strains believed to be of the same species or at least very closely related one to another.

Each fermentation is a problem of its own, and the choice of analytical methods must vary with the group of bacteria under investigation. To give specific directions here for even the most common contingencies would consume an inordinate amount of space. Except in one instance (action on nitrates) which seems nowhere to have been treated adequately, only the main features will be considered here. Of the various compilations of methods, the three following may be particularly useful for purposes of reference: A.O.A.C., Official and Tentative Methods of Analysis, 5th Ed. 1940; Abderhalden, E., Handbuch der biologischen Arbeitsmethoden (Urban, Berlin); and Peters and Van Slyke (1931, 1932).

Preparation of Bacterial Juices

Cell-free juices prepared from bacteria are receiving increasing use in physiological studies and are serving in the elucidation of problems dealing with mechanism of bacterial action on substrates. Juices are obtained usually by one of the following methods: (a) Extraction of juice, (b) press juice, (c) filtrates, (d) grinding, or a combination of methods. The Booth-Green (1938) mill has been used to good advantage; in the United States, the powdered glass-grinding-extraction technic has given good results. The Booth-Green mill is unobtainable at present; it has been used especially by the English workers. In general the technic of grinding with powdered glass, followed by extraction, has certain advantages both in cost of equipment and breadth of application. Bacteria are grown in liquid culture, centrifuged in a Sharples super-centrifuge at 30,000 r.p.m. and the resulting paste mixed with a quantity of powdered glass (generally two parts paste: 1 part glass) with a particle size of about 2 μ. The powdered glass is prepared by grinding clean pyrex in a ball mill with steel balls for one hour. A mask should be worn. The bacteria-glass mixture is forced through a grinding apparatus comprising two glass cones, one turning within the other. The bacteria are cut by the fine glass particles. See: Wiggert, et al (1940); Werkman and Wood (1940). The mixture is extracted with water or a buffer solution, and after that is centrifuged to throw down the glass. The extract then may be centrifuged in a Beams air-driven centrifuge until clear. A differential separation of enzymes may be accomplished by the Beams centrifugation. The supernatant liquid may be dialyzed through collodion or cellophane membranes to remove coenzymes and inorganic ions. Juices are desirable when separate enzyme systems are under investigation; also when the cell wall is impermeable to a substrate,

particularly in the case of an intermediate product which is formed within the cell.

Relation to Free Oxygen

A section of Leaflet V having the same heading as this describes methods for distinguishing roughly between aerobes and anaerobes. For a careful physiological study of any organism one must realize, however, that such determinations as those mentioned in Leaflet V are quite incomplete. It is especially to be observed that the rough methods given there do not distinguish between strict anaerobes and microaerophilic organisms. For a more adequate study of the relation of an organism to free oxygen, there are two points in particular which require careful investigation: first, the optimum oxygen tension (which may be considered in the case of anaerobes from the standpoint of oxygen tolerance); second, the respiratory quotient.

Optimum Oxygen Tension. Vessels large enough to furnish an adequate oxygen supply must be used. Probably the best method is to place the cultures growing on liquid or agar as desired, in a Novy jar, to evacuate and to replace the air with a mixture of gases containing a known percentage of oxygen. Such a method is well adapted to determining oxygen tolerance of microaerophilic organisms. It should be particularly remarked that the 'absence' of oxygen in the gas space over the bacterial culture should be tested for directly by employment of a suitable indicator (e.g., solution of reduced methylene blue or indigo disulfonate) properly applied to the gas phase. Use of the indicator within the medium is of uncertain value.

Respiratory Quotient. A similar apparatus may be used if provided with stop-cocks to allow the removal of samples of gas for analysis. A manometer should always be present on such a system to show changes in gas pressure. In the sample of gas removed, the carbon dioxide may be determined by absorption with standard alkali, after which the oxygen may be removed by alkaline pyrogallol. The respiratory quotient is obtained by dividing the volume of carbon dioxide produced by that of the oxygen consumed.

Details of these methods are not given here and must of necessity be varied with the organisms under investigation. A useful set-up for determining the points above mentioned is described by Soule (1928). Attention should also be called to the manometric technic for physiological studies on microörganisms. This technic provides a powerful method of attack and should find wide use in bacteriological research. It offers the most convenient and accurate method available for following reactions in which gas is evolved or taken up, and has been extended to include chemical determination of products. The manometric technic has been used successfully in studies determining rates of reaction (especially when CO_2 is evolved, O_2 taken up, or acids formed), vitamin or growth factor requirements, CO_2 utilization, and efficacy of disinfectants. Manometric methods can be adapted to a wide variety of uses. For general purposes in physiological bacteriology, the Warburg type of manometer is used. This

is a constant volume type in which the reaction flask is attached to a U-shaped manometric tube. The change in pressure on the liquid in the tube is read, from which the O_2-uptake and CO_2 evolved are easily calculated. Anaerobically, CO_2 and H_2 are readily determined. The manual by Dixon (1934) may be consulted for theoretical and manipulative details.

Cleavage of Carbohydrates, Alcohols, and Glucosides

Under this heading in Leaflet V are given the most common routine tests, designed merely to show whether or not an organism produces acid or gas in certain standard media. Such tests are valuable, but do not give a sufficient idea as to the action of the organism on the carbon compound under investigation. In a comprehensive physiological study, various more detailed methods are necessary. The present leaflet is designed to indicate a few of these methods.

Choice of Carbon Compounds. The carbon compounds employed in a study of this sort should be of the utmost purity. A considerable variety of such compounds is now available. It is not always necessary to use all of them; but for many groups of bacteria it will be known in advance which may be expected to give the most useful information. The following list gives the compounds most frequently used in fermentation studies:

Monosaccharides:	Pentoses: l-arabinose, xylose, rhamnose
	Hexoses: glucose, fructose, mannose, galactose
Disaccharides:	Sucrose, maltose, lactose, trehalose, cellobiose, melibiose
Trisaccharides:	Raffinose, melezitose
Polysaccharides:	Starch, inulin, dextrin, glycogen
Alcohols:	Trihydric: glycerol
	Tetrahydric: erythritol
	Pentahydric: adonitol, arabitol
	Hexahydric: mannitol, dulcitol, sorbitol
Glucosides:	Salicin, coniferin, aesculin

Several of these compounds are hydrolyzed or otherwise decomposed at the temperature necessary for sterilization. For careful work, therefore, such compounds must be sterilized separately, by Berkefeld filtration or by autoclaving in concentrated (ordinarily 20%, unless the viscosity is too great), slightly acid (pH6.8) aqueous solution, and added aseptically to the basal medium. In the latter case, autoclaving for 15 minutes at 15 pounds pressure and plunging into cold water has proved useful. Sugars are particularly subject to chemical change in the presence of phosphates or in alkaline solution.

Ordinarily a concentration of 1% in the medium is satisfactory; but one can often economize (in the case of expensive compounds) by employing lower concentrations.

Choice of a Basal Medium. There are many bacteria that will not grow in beef extract agar or broth, and modifications are necessary in order to secure sufficient growth to determine whether or not utiliza-

tion of the added carbon compound can occur. Often the poor growth may be due to the lack of necessary inorganic salts or to some unknown organic factor in the peptone which is required by many bacteria. Probably the most satisfactory way to supply the latter factor is thru the use of yeast extract. (See yeast extract broth, p. II14-5, Leaflet II.) This furnishes a satisfactory basal medium in studying propionic acid bacteria, streptococci or lactobacilli. In the case of some microaerophiles better growth may be secured by employing a semisolid agar as a basal medium (see p. II14-5). Some bacteria, on the other hand, fail to grow on standard broth or agar because of the presence of too much organic matter. For them the ammonium phosphate medium (liquid or agar) given on page II11-15 will often give satisfactory results. This synthetic medium must be used with a little caution, however, as it is poorly buffered and quite a high final H-ion concentration (e.g., pH 5) may not necessarily mean acid production from the carbohydrate (see discussion three paragraphs below).

It is often necessary to prevent an appreciable rise in H-ion concentration. This is ordinarily accomplished by adding an excess of sterilized $CaCO_3$ to each culture tube or flask, or by suitable buffering of the medium.

If calcium carbonate is used, it should be a fine powder so as to provide great surface for neutralization of the acids formed. In addition the carbonate should be suspended throughout the medium by adequate agitation, otherwise calcium carbonate is not a very effective neutralizing agent.

Analytical Methods. In a study of fermentation, the following determinations are commonly made: Final H-ion concentration, residual sugar, kinds and quantities of organic acids, neutral solvents, carbon dioxide. The choice as to which of these determinations to make and sometimes as to what methods to employ must often depend on the organism or group of organisms under investigation. In a complete study it is necessary to account for the carbon originally present in the substrate (usually a carbohydrate). This carbon should theoretically be accounted for among the products of fermentation. Likewise the state of oxidation of the products should equal that of the substrate, indicated by the redox index. The use of the redox index is extremely useful in careful fermentation studies as a measure of the accuracy of results. For a discussion see Johnson, Peterson and Fred (1931).

Final H-ion Concentration. This may be determined colorimetrically or electrometrically according to the accuracy desired and the applicability of the method to the conditions of the experiment. The colorimetric method is given in Leaflet IX. Standard texts, like Clark's "The Determination of Hydrogen Ions" 3rd Ed., should be consulted for the electrometric method; the use of the glass electrode has recently found marked favor (see Leaflet IX, p. IX15-7.)

In interpreting results, the buffer content of the medium must be taken into consideration. The final reaction is the resultant of various factors including the following: production of fatty acids, of CO_2, of ammonia (or other basic substances) from nitrogenous matter present; withdrawal of either cation or anion from mineral salts with con-

sequent freeing of acid or base. Accordingly, direct comparisons between results in different basal media should not be made.

Residual Sugar. Determination of sugar in cultures and in uninoculated controls may be made by the method of Shaffer and Hartmann (1921) or its modification by Stiles, Peterson, and Fred (1926). Both are iodometric modifications of the Fehling procedure. In using this analytical method it is important that the medium contain only a little more sugar than the bacteria can use. The method has its greatest accuracy only within certain limits, so it is important that wherever possible the amount of reducing sugar in the aliquot lie within those limits. Accordingly, preliminary determinations with varying percentages of sugar are often necessary before deciding on the most suitable concentration or the most satisfactory volume to employ for an aliquot.

It is understood that the method is not as accurate in media containing beef broth as in solutions that are free from it. It cannot be used in the presence of nitrites; but these may first be removed by heating in the presence of urea and acid.

Quantity of Acid Produced (Titratable Acidity). Titration of an aliquot sample of a culture with standard alkali to an arbitrarily chosen end-point (usually phenolphthalein or phenol red) is often employed (after deduction of corresponding blank titration value) as a measure of the quantity of acid products present. The sample may be boiled before titration if it is desired to exclude CO_2 from the determination. The results are most directly expressed in terms of normal acid, or as milliliters of N/10 acid per 100 ml. of culture. They are sometimes expressed presumptively in terms of the predominant organic acid (e.g., lactic acid) assumed to be produced by the bacteria.

Nature of Acids Produced. To neutralize the acids produced, an excess of $CaCO_3$ may be added to the medium (see p. VI42-6). Or if it is not desirable to have carbonate present an indicator may be added and sterile NaOH introduced aseptically from time to time from a container sterilized with the culture flask. Incubation should continue to completion.

The acids most frequently present are: (1) the volatile fatty acids, formic, acetic, propionic and butyric; (2) the non-volatile acids, lactic and succinic. Separation of the volatile acids is ordinarily effected by steam distillation after acidification with H_2SO_4 to pH 2.0 to liberate the acids. It is necessary to collect twelve volumes of distillate; e.g., 300 ml. from 25 ml. of medium, in order to remove the volatile acids quantitatively. The non-volatile acids are recovered from the residue of the steam distillation by continuous extraction with ether for 48 hours.

Lactic acid may be determined in the extract by oxidation with permanganate to acetaldehyde. The aldehyde is bound in bisulfite and the bound bisulfite determined iodometrically (Friedemann and Graeser, 1933). The succinic acid may be precipitated as the silver salt and weighed, or the silver of the salt determined volumetrically (Moyle, 1924).

The volatile fatty acids frequently consist of formic and acetic

acids. In this case the total volatile acid in the distillate may be determined by titration, and the acetic acid calculated by difference. The formic acid may be determined by oxidation with $HgCl_2$ and the resulting HgCl weighed (Auerbach and Zeglin, 1922). The Duclaux distillation method as modified by Gillespie and Walters (1917), or Virtanen and Pulkki (1928), or the partition method of Osburn, Wood and Werkman (1933), (1936), may be used for quantitative determination of more complex mixtures.

The partition method is applicable to the quantitative estimation of mixtures of formic, acetic, propionic and butyric acids, and the qualitative detection of other acids. The basis of the method is the characteristic distribution of an acid between water and an immiscible solvent, such as ethyl ether, when the two are vigorously shaken together.

Pyruvic Acid. A qualitative test is finding increasing use. The test is not absolutely specific for pyruvic acid but under the conditions used in bacteriology is of qualitative significance.

Pyruvic acid may be determined qualitatively by a blue color produced with Na nitroprusside (Simon and Piaux, 1924). Two milliliters of the solution containing pyruvic acid are saturated with $(NH_4)_2SO_4$, 4 drops of a 2% nitroprusside solution are added, plus 1 ml. conc. NH_4OH. After a few minutes, a blue color is produced, specific for pyruvic acid (and acetophenone). Other ketone compounds, such as acetone, acetoacetic ester, acetoacetic acid, creatinine, and glutathione give color reactions varying from orange to red to purple.

Pyruvic acid may be determined quantitatively either by the reaction with ceric sulfate, or salicylaldehyde. With the ceric sulfate method, pyruvic acid is oxidized to acetic acid and CO_2. The CO_2 released can be determined manometrically, or the excess of Ce^{++++} can be determined titrimetrically with $FeSO_4$. Lactic acid will interfere when present in large amounts. The reaction is specific for alpha keto acids. (Fromageot and Desnuelle, 1935.)

Pyruvic acid may be determined colorimetrically by reaction with salicylaldehyde plus strong KOH. Oxalacetic acid will not interfere. (Straub, 1936.) Less than 0.1 mg. of pyruvic acid can be determined accurately by this method. Acetaldehyde and acetone also give a color reaction.

Succinic Acid may be precipitated as the silver salt and weighed, or the silver of the salt may be determined volumetrically (Moyle, 1924.) The acid may also be determined quantitatively by the use of an enzyme obtained from beef heart. Succinic acid is extracted from solution with ether and determined by measuring the oxygen necessary for oxidation of succinate to fumarate in the presence of the enzyme. (Gözsy, 1935). One mole of O_2 taken up is equivalent to two moles of succinic acid. The preparation of the enzyme is described by Weil-Malherbe (1937) and Krebs (1937). Potter and Elvehjem (1936) describe a simple mechanical modification to replace grinding with sand.

Substances which will be oxidized by this enzyme preparation are

succinate, methyl succinate (Thunberg, 1933), α-glycerophosphate (Green, 1936) and d-glutaric acid (Weil-Malherbe, 1937). Methyl succinate has not been found in biological material and α-glycerophosphate and d-glutamate are not extracted with ether, therefore, this method is highly specific for succinic acid (Krebs, 1937).

Neutral Solvents (*acetone and ethyl, butyl and isopropyl alcohols*). These solvents are best distilled from a neutral or slightly alkaline fermentation liquor. Acetone, in an aliquot of the distillate, is oxidized with iodine in alkali and excess of the iodine back-titrated with thiosulfate. (Goodwin 1920). The other solvents are not oxidized under these conditions. Isopropyl alcohol can be oxidized by dichromate and orthophosphoric acid to acetone and the latter distilled off and determined as above. Stahly, Osburn and Werkman (1934) show that 94% of the acetone is recovered in the distillation. The analytical results should, therefore, be corrected accordingly. These authors state that *ethyl alcohol* can be entirely oxidized by dichromate to acetic acid, while in the case of *butyl alcohol*, 89.6% is oxidized to butyric acid and 10.4% to acetic acid. The two acids may then be determined by distillation or by partition. For small quantities of ethyl or butyl alcohol the method of Johnson (1932) may be used.

Carbon Dioxide. Large quantities of CO_2 may be detected by the appearance of gas and its characteristic reactions. Because of its great solubility, however, it can seldom be thus detected, and a measure of the gas evolved is never an accurate determination of the quantity of CO_2 produced.

For accurate results, use should be made of an aeration train of which the essential elements are: a wash tower containing alkali to remove CO_2 from incoming air; a flask or other container for the culture; an absorption tower containing a measured amount of standard alkali with beads or other device to break up the stream of air; and an aspirator or pump to force or to pull the air thru the train. When using this method special precautions to avoid contamination should be observed; and no reliance should be placed on results unless tests at the end of the experiment show that the original organism is present in pure culture.

When a considerable number of cultures are to be studied simultaneously, the Eldredge tube (Fig. 1) can be used more conveniently and often with sufficiently accurate results. A satisfactory sized tube is one having a capacity of about 60 ml. in each arm. (These tubes are not as yet listed by supply houses, but arrangements to handle them have been made with the Will Corp., Rochester, N. Y. and Macalaster Bicknell Co., Washington and Moore Sts., Cambridge, Mass.)

In using the Eldredge tube, place 20 ml. of the medium in one of the horizontal arms and sterilize. Inoculate and then place in the other arm a measured quantity (usually 15 to 25 ml., depending upon the amount of CO_2 expected) of a freshly prepared N/10 barium hydroxide solution. (One may use NaOH or KOH, but the insolubility of the $BaCO_3$ formed makes $Ba(OH)_2$ more satisfactory in giving a visual indication of CO_2 production.) Immediately after inserting the alkali, push the cotton plugs down in the tubes and seal. After at least two weeks incubation titrate the barium hydroxide with N/10 HCl or preferably H_2SO_4, using phenolphthalein as an indicator. Compute the amount of CO_2 produced from the equation: ml. of $Ba(OH)_2 \times$ normality of $Ba(OH)_2$

$\times 0.022$ = grams of CO_2 (i.e., 1 ml. of N/10 $Ba(OH)_2$ converted into the carbonate represents 0.0022 g. CO_2.)

The contents of the culture arm of the Eldredge tube may be analyzed, if desired, to show the amount of sugar remaining, by the methods given above (p. VI$_{42}$-7). One can strike a balance between the CO_2 given off and the sugar-carbon consumed, and thus decide whether to look for other end-products. This makes the Eldredge tube method a useful preliminary in some cases for a more extensive study of the fermentation.

Hydrogen. Hydrogen is usually determined in one of two ways: by measurement of volume; by combustion to water and determination of the water by weight. In the first procedure the gas produced

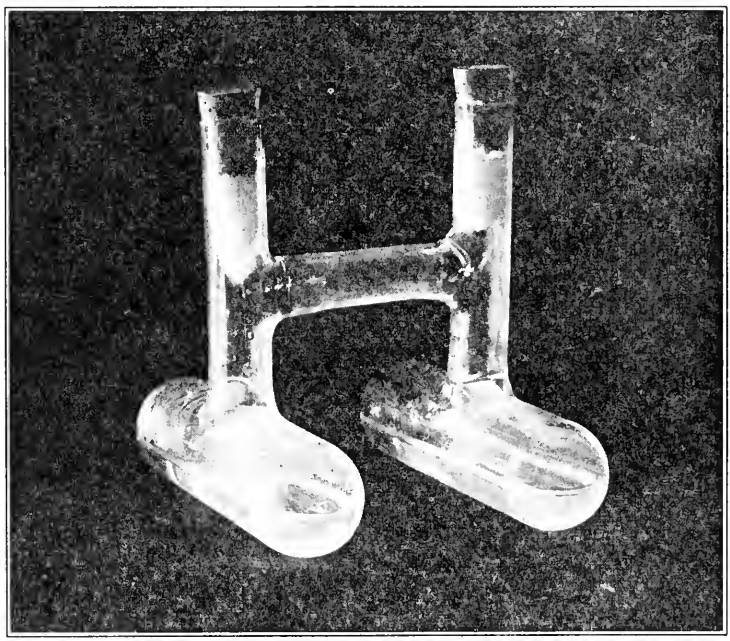


FIG. 1. THE ELDREDGE TUBE
Height 4″, width 3½″, length horizontal tubes, 4½″.

in the fermentation may be collected over alkali, and in the absence of other gases such as methane, the hydrogen measured directly. Also the gas may be exploded in a Hempel pipette, and the decrease in volume of gas measured. When methane is present, the CO_2 formed by its combustion must be measured and a correction applied (cf. Gas Chemists' Handbook, 1929; McCulloch, 1938).

The combustion of hydrogen to water may be brought about by CuO at 250° C. Methane is not oxidized under these conditions. The water is collected in a suitable train and weighed.

Acetyl-methyl-carbinol. A minor by-product, which has come into prominence because of its detection in the Voges-Proskauer test for

distinguishing between the members of the colon group, is acetyl-methyl-carbinol ($CH_3 \cdot CO \cdot CHOH \cdot CH_3$). The Voges-Proskauer test is described in Leaflet V (p. V41-20). A method for accurately detecting this compound, originally proposed by Lemoigne (1913) has been improved and described in detail by Kluyver, et al (1925). The procedure depends upon oxidation to diacetyl ($CH_3 \cdot CO \cdot CO \cdot CH_3$), distillation and precipitation in the form of nickel dimethylglyoxime, which shows as characteristic reddish crystals. Stahly and Werkman (1936) show that approximately 84% of the acetyl-methyl-carbinol may be thus determined.

2,3-Butylene Glycol. A further common by-product in the case of organisms of the groups that frequently show the presence of acetyl-methyl-carbinol is 2,3-butylene glycol. A method of determining this, depending upon oxidation to acetaldehyde and subsequent titration of the HCl formed by the reaction between the acetaldehyde and hydroxylamine hydrochloride, has been developed by Brockmann and Werkman (1933). The following method is a modification of that of Brockmann and Werkman.

Sugar interferes in the alkaline distillation, and if present must be removed prior to analysis. This is accomplished by the $CuSO_4$-lime method of Hewitt. (Hewitt, 1932). The liquor to which has been added the copper-lime reagent is brought to definite volume and centrifuged. The supernatant is decanted and filtered. This method also removes citric acid.

An aliquot of the sample is made alkaline to phenolphthalein and anhydrous Na_2SO_4 added. (10 g. Na_2SO_4 for 50 ml. aliquot). The solution is directly distilled (in a Kjeldahl flask of convenient volume) to saturation (20 ml.), and 14 volumes (280 ml.) removed by steam distillation. The distillate is made up to definite volume, and an aliquot, containing not more than 0.6 mM of glycol, removed, and 6 ml. of a potassium periodate reagent (5.75 g. KIO_4 dissolved in 100 ml. 3.6 N H_2SO_4) is added.

Distill into 10 ml. fresh 1% $NaHSO_3$, with the end of the adapter beneath the surface of the $NaHSO_3$ solution. Destroy excess bisulfite by adding 0.25 N I_2, with starch indicator. Destroy aldehyde-bisulfite complex by adding excess $NaHCO_3$, (0.5-1 g.) and titrate the liberated bisulfite with weak (0.05N) I_2, using starch indicator (Friedemann and Graeser, 1933). Compute the amount of 2,3-butylene glycol from the equation: ml. of $I_2 \times$ normality of $I_2 \div 4 =$ conc. of butylene glycol in millimols; (i.e. 1 ml. 0.05N $I_2 = 0.0112$ g. butylene glycol).

If acetyl-methyl-carbinol is present, an abnormally high glycol value results. Acetyl-methyl-carbinol must be determined in the distillate, and one-half the value obtained subtracted from the uncorrected glycol value. (Stahly and Werkman, 1936).

Interfering Reactions. Many of the methods of analysis may result in serious error, owing to their lack of specificity. Each type of fermentation requires a careful selection of methods. The following are a few examples of interference.

Acetyl-methyl-carbinol is oxidized by $CuSO_4$ in the determination of reducing sugars (cf. Stahly and Werkman, 1936, and Langlykke and Peterson, 1937, for correction factors).

Approximately 5% of the lactic acid volatilizes during steam distillation of the volatile acids. When lactic acid is present in large amounts, the volatile acids should be neutralized, evaporated to a small volume (25–50 ml.), acidified with H_2SO_4, and again steam distilled. This procedure eliminates most of the lactic acid from the distillate. Thirty per cent of pyruvic acid volatilizes; usually three distillations are necessary to eliminate this acid from the distillate. It is, perhaps, better to determine the volatilized pyruvic acid by ceric sulfate oxidation (Fromageot and Desnuelle, 1935) or by the iodoform reaction (Wendel, 1932) and apply a correction for this acid.

Acetone is usually determined by the iodoform reaction. Any other neutral volatile compound which gives the iodoform reaction will, of course, interfere with this method, particularly acetyl-methyl-carbinol, nearly 60% of which volatilizes during a half volume distillation. The acetyl-methyl-carbinol in the distillate can be determined as nickel dimethyl-glyoximate and a correction applied, or the procedure of Langlykke and Peterson (1937) may be used.

Acetyl-methyl-carbinol and 2,3-butylene glycol interfere in the determination of lactic acid. They may be removed by alkaline steam distillation (14 volumes) from a solution saturated with Na_2SO_4. The lactic acid is determined on the residue of distillation. When sugars are present, alkaline distillation causes caramelization and consequently, interference with both the glycol and lactic acid determinations. Separation of the glycol from the sugar and lactic acid may be accomplished by extraction of an alkaline solution with ether continuously for 72 hours. The glycol is recovered in the extract. The interference of sugar may also be avoided, without extraction, by removing the sugar by copper-lime treatment (Hewitt, 1932) and then making an alkaline distillation.

Determination of Dehydrogenases

The determination of the presence of a specific dehydrogenase may be made by the Thunberg technique (methylene blue reduction). There are many modifications of this procedure (e.g. Hopkins and Dixon, 1922; Yudkin, 1933). These modifications are concerned with methods of obtaining anaerobic conditions and amounts of reactants.

The essential points of the procedure are:

1. To have a glass tube with a side arm or hollow stopper in which anaerobic conditions can be maintained.
2. A constant temperature water-bath.
3. An adequate buffer.
4. An accurate control.

Anaerobic conditions may be obtained by vacuum, vacuum followed by oxygen-free nitrogen, or by oxygen-free nitrogen alone. If the latter is employed, the apparatus should be arranged to allow bubbling of nitrogen through the reagents for a few minutes.

A constant temperature water-bath is essential to bring the reactants quickly to the desired temperature and to maintain that temperature throughout the experiment.

The buffer must be carefully selected as to type of buffer and concentration. One must have sufficient buffer to maintain the desired pH throughout the duration of the experiment.

The standard (90% reduction) may be prepared by substituting distilled water for the substrate and by adding 0.1 the regular amount of methylene blue and leaving it open to the air.

The reactants may consist of 1 ml. each of buffer, substrate (N/10), methylene blue solution (1/5,000) and the bacterial suspension. The buffer, substrate and methylene blue are mixed together. The suspension is placed in the side arm or in the hollow stopper. The system is made anaerobic and placed in the water-bath at a predetermined temperature, usually 30°, 37° or 40°C. When the temperature has reached that of the water-bath, the suspension is mixed with the other substances and the time recorded. The time required by the substrate (H_2-donator) to reduce the methylene blue, until the color matches that of the standard, is compared to the endogenous reduction time; the latter is the time required by the suspension to reduce the methylene blue in the absence of the substrate.

A dehydrogenase is considered present when the reduction time in the presence of substrate is less than the endogenous time.

Cleavage of Proteins and Their Products

The liquefaction of insoluble nitrogenous organic material such as gelatin, coagulated casein or blood serum is one criterion of the cleavage of these substances. As the process continues, progressive changes occur in the biuret reaction and in the number of "free" amino and carboxyl groups. In addition, there appear certain more or less characteristic end-products, such as ammonia, hydrogen sulfide, mercaptans, and tyrosine (depending on the constitution of the nitrogenous substrate) which are often readily perceptible.

The Biuret Reaction. Proteins form colored complexes with cupric ions in alkaline solution. This is one of a general type of reactions by ammonia or substituted ammonias. The color of the complex is violet with the more complex polypeptides and proteins, and pinkish lavender with peptones.

The test is carried out by making the culture solution alkaline (about molar) with NaOH and then adding 0.1% $CuSO_4$ dropwise until the minimum amount has been added to produce the pink to violet color. Ammonium salts interfere and, if present, should be removed before testing.

Amino Nitrogen. The commonly employed measures of amino compounds are the Sörensen formol titration and the well-known Van Slyke procedure.

The Formol Titration: This method depends on the increase in acidity brought about when neutralized formaldehyde is added to a solution containing ammonia, primary amines, amino acids or polypeptides. A practical procedure is given by Brown (1923).

To 1 volume of the culture fluid add 9 volumes of water and bring the reaction to pH 8 by the addition of N/20 NaOH or HCl. Add 8 volumes of formaldehyde solution (approximately 40% formaldehyde). Immediately titrate the mixture to pH 8 with N/20 NaOH. Determine the amount of N/20 NaOH required to bring 8 volumes of the formaldehyde solution to pH 8 and subtract this from the titration of the mixture. The result is the formol titration expressed as ml. of N/20 NaOH per 100 ml. of the culture fluid.

Deductions as to the amino-N content of the fluid should take into account the ammonia included in the determination.

The Van Slyke (1913) Amino-N Method: This procedure depends upon the production of gaseous nitrogen when nitrous acid acts on an aliphatic amine. Special apparatus is required. The recently introduced (Van Slyke, 1929) manometric method is more generally useful than the older volumetric. For details of the procedure consult the original references (also, Peters and Van Slyke, 1932, 385).

Ammonia. The quantitative determination of ammonia must be carried out by a procedure which will not decompose potential ammonia-producing compounds. From this standpoint, the Folin (1902) aeration method as modified by Van Slyke and Cullen (1916) is probably safer than the usual distillation from a solution treated with MgO. The procedure involves a cautious aeration of the alkalinized solution with ammonia-free air into standard acid which is subsequently titrated.

H_2S and Volatile Mercaptans. Aeration of the acidified culture fluid with H_2S-free air and absorption of the volatile sulfides in a solution of zinc or lead acetate would be the first step. Oxidation of the sulfide with Na_2O_2 would produce sulfate which is precipitable as $BaSO_4$; oxidation of the mercaptans would produce sulfonic acid which is not precipitable as $BaSO_4$. Intensive oxidation in the presence of nitrate and chlorate would convert all of the sulfur to sulfate. These are the general principles upon which a method of analysis can be based.

Action on Inorganic Nitrogenous Compounds

There are many bacteria that are capable of utilizing inorganic sources of nitrogen, such as nitrates or ammonium salts. Some utilize such nitrogen sources in preference to organic forms, others in addition to the latter. When action on such compounds occurs it is of value to make more of a study of it than is given in Leaflet V under "Action on Nitrates".

Action on Ammonium Salts. There are a few bacteria that utilize ammonium salts when furnished with no other source of nitrogen. In such cases it is frequently of value to determine what percentage of the ammonia furnished is used by the organisms. For such purposes the ammonia can best be determined by distillation with magnesium oxide and collection of the ammonia in standard acid solution, in which it can be determined by titration.

Action on Nitrates. The most common action of bacteria on nitrates is one of reduction to nitrite, to ammonia, or to free nitrogen, or pos-

sibly to all three. Apparently reduction often accompanies or is preliminary to utilization of nitrate by bacteria, and confusion often arises in not distinguishing between the two processes. Thus, if an organism is furnished with either nitrate or nitrite and that compound disappears, one sometimes finds the statement made that it has been reduced, when it may well have been utilized without reduction.

Before beginning the study of any organism in regard to this point, one must first decide the object of his study—whether he merely wishes to make a test for diagnostic purposes in identifying his culture with some published description, or desires to know just what the organism actually does to nitrate. In the former case he must remember that if an organism has been described in the literature as reducing or not reducing nitrate, such a statement ordinarily means that it does or does not produce nitrite in a nitrate medium. For diagnostic purposes, therefore, one can ordinarily get along with a nitrite determination alone, if the test is made under the proper conditions and is properly interpreted. This use of the nitrate reduction test for diagnostic purposes alone is discussed in Leaflet V (page V$_{44}$-9).

If, on the other hand, the investigator desires to know the real action of his culture on nitrate, a series of tests is often needed, since a negative result is meaningless unless supported by evidence from other tests. In case of a negative nitrite test, several possibilities are to be considered: 1) nitrite may be demonstrable if some other nitrate medium is used[1]; 2) nitrate may be utilized by the bacteria without reduction; 3) nitrates may be reduced to ammonia or free nitrogen without accumulation of nitrite in detectable quantity; 4) no action on the nitrate may have occurred. Methods for determining which of these explanations applies have been recently discussed by Conn (1936). To make this determination often means a small research problem in the case of any organism under investigation. Tests called for in such an investigation are as follows:

Qualitative tests for nitrate: (good only in the absence of nitrite.)
Zinc dust test: See Leaflet V, p. V$_{44}$-10.
Diphenylamine test: See *idem*.

Quantitative test for nitrate: This is necessary if it is desired to know whether the nitrate has been partially consumed even tho no end-products can be detected. For details of procedure see Methods of Analysis of the A.O.A.C. (1934) Chapter XXXVII, Sec. 16 and 17.

Qualitative test for ammonia: (Significant only if the organism has been growing on a synthetic medium with no nitrogen source other than the nitrate.) The Thomas test as employed by Hucker and Wall (1922) may be employed; but the modification of this test described by Hansen (1930), using hypobromite instead of hypochlorite, seems to be more reliable.

Test for nitrite consumption: In instances when it is suspected that nitrite may be consumed as rapidly as it is formed from the nitrate, Bronfenbrenner and Schlesinger (1920) inoculate the organisms in question into a medium containing only 2 p.p.m. of potassium

[1]Thus ZoBell (1932) finds semi-solid agar (0.3%) invaluable in studying nitrate reduction of the Brucella and Salmonella groups.

nitrite and after incubation apply the regular nitrite test. It is assumed that an organism not able to destroy this small amount of nitrite cannot destroy or consume it as rapidly as it may be produced from nitrate.

The matter of action on nitrates can well be summarized by listing a series of questions that must be answered before this action can be thoroly understood. These questions are:

1) Does the organism utilize completely 1% of KNO_3? In what media?

2) If not, does it utilize part of the KNO_3 furnished? What percentage?

3) If either of these questions are answered in the affirmative, does the organism actually *reduce* nitrate? Before answering this question the following subordinate questions must be answered:

a) Does it produce nitrite or gas in a nitrate medium but not in the same medium without nitrate?

b) Does it produce ammonia in a nitrate medium containing no source of nitrogen other than the nitrate?

c) If both these questions are answered in the negative, is the organism able to destroy 2 p.p.m. KNO_2 in a medium in which it is being studied? If so, it may still be a nitrate-reducer, the accumulation of nitrite being prevented by its action on nitrite. It must not however, be assumed to reduce nitrite as it may utilize it as a source of nitrogen without reduction, or may conceivably convert it to nitrate.

Recommendations: It must be distinctly understood that the Committee does not recommend making such a study as the above to determine the action on nitrates in securing data for routine descriptions of organisms. For such routine purposes the tests given in Leaflet V are ordinarily sufficient; but it is emphasized that in recording or publishing negative results of such tests one must not make the statement "nitrates not reduced". A negative nitrite test should merely be recorded "no nitrite produced from nitrate under the conditions of the experiment".

The further tests outlined in this Leaflet are to be regarded as research methods to be employed when information is desired concerning the true action on nitrate of any organism under investigation.

Action on Erythrocytes

Certain organisms during their growth cause a number of changes in the pigment of red blood corpuscles. Some organisms break down the corpuscles, liberating the hemoglobin, due to the action of hemolytic substances. Some of these substances are analogous to exotoxins and can be found in the filtrate of broth cultures. Other organisms change the hemoglobin in the cells to methemoglobin or sulfhemoglobin, producing a greenish coloration. While these organisms are

intact, the erythrocytes are not hemolyzed. Later, when the bacteria break down, substances are liberated which have a more or less pronounced hemolytic action. A third group of organisms are "indifferent," producing no visible change in the hemoglobin or erythrocytes. The production of hemolysins and changes occurring in the hemoglobin under bacterial action are important in the differentiation of streptococci, pneumococci and other bacteria. *Streptococcus pyogenes* is the type of organism which produces an exohemolysin; pneumococci and streptococci of the *viridans* group, are types of organisms which produce methemoglobin.

Method I. Blood Agar Plate Method. Either streak cultures on blood agar plates or poured plates of blood agar mixed with bacteria can be used for this purpose. The sharpest results are obtained with poured plates. For the streak method, prepare blood agar plates by melting 100 ml. of 2% meat infusion agar, cooling the agar to 45° C., adding 5–10 ml. of sterile defibrinated blood (sheep, rabbit or horse blood) and pouring this blood agar into Petri dishes. After the agar has hardened, streak the surface with the organism. Incubate the plate for 24 hours or longer at 37° C. Also incubate uninoculated plates as checks against contamination. A clear area under and beyond the edge of the growth (beta hemolysis) indicates laking of the red cells due to an hemolysin elaborated by the organism. Organisms which produce methemoglobin cause a greenish coloration (alpha hemolysis) in the blood adjacent to the growth. In using the poured plate method, the blood agar is prepared in a tube or flask and inoculated with a suspension of the organisms that will give 25 to 50 colonies per plate. It is important that no sugar be added to the agar. The temperature at the time of mixing the organisms with agar should be approximately 45° C. The inoculated blood agar is poured into Petri dishes, allowed to harden and incubated. After incubation, clear areas, having varied significant characteristics, appear around the colonies which produce hemolysin (beta). The colonies of "green producing" streptococci and pneumococci appear surrounded by a greenish zone of erythrocytes containing methemoglobin (alpha). After continued incubation of this type of culture, a zone of hemolysis occurs beyond the zone of greenish cells, and at times several rings of alternate hemolysis and methemoglobin formation may be observed.

Method II. Blood Broth Mixtures. To 0.5 ml. of a sterile 5% suspension of washed rabbit, sheep or horse blood cells in 0.85% NaCl solution, add 0.5 ml. of a 12 to 18 hour sugar-free broth culture of the organism to be tested. Incubate this mixture for 2 hours, at 37° C., preferably in a water bath. The production of an hemolysin is shown by the laking of the cells, giving a clear solution. Organisms which form methemoglobin produce darkening of the cells, and do not hemolyze them in this test. A tube containing 0.5 ml. each of the blood suspension and of sterile broth should be inoculated as a control. The corpuscles of rabbits blood are removed by centrifuging and washed as described on p. VIII$_{10}$-15 of Leaflet VIII.

For hemolytic streptococci, the addition of serum to the broth enhances hemolysin production.

Method III. Filtrates. The hemolysin produced by some bacteria occurs free in the broth in which the organism has been growing. Its presence can be demonstrated by adding a sterile filtrate (Berkfeld or Seitz filtrate) of the 12 to 18 hour culture to a 5% suspension of the sterile blood or of suitable washed erythrocytes.

Excessive exposure to air may inhibit or destroy the hemolytic activity of the filtrate. (See Shwachman, Hellerman, and Cohen, 1934.)

REFERENCES

American Gas Association. 1929. Gas Chemists' Handbook, *3rd. ed.* 725 pp. New York.

Association of Official Agricultural Chemists. 1940. Official and Tentative Methods of Analysis, *5th ed.* Washington, D. C.

Auerbach, F. and Zeglin, H. 1922. Beiträge zur Kenntnis der Ameisensäure. Zts. physik. Chem., **103**, 161–77.

Booth, V. H. and Green, D. E. 1938. A wet crushing mill for microörganisms. Biochem. J., **32**, 855–61.

Brockmann, M. C. and Werkman, C. H. 1933. Determination of 2, 3-butylene glycol in fermentations. Ind. Eng. Chem., Anal. Ed., **5**, 206.

Brown, J. H. 1919. The use of blood agar for the study of streptococci. Monograph No. 9, Rockefeller Institute for Medical Research.

Bronfenbrenner, J. and Schlesinger, M. J. 1920. A study of nitrate reduction by bacteria. Abstr. Bact., **4**, 2.

Brown, J. H. 1923. The formol titration of bacteriological media. J. Bact., **8**, 245–67.

Conn, H. J. 1936. On the detection of nitrate reduction. J. Bact., **31**, 225–33.

Dixon, M. 1934. Manometric Methods. The Macmillan Co., New York.

Folin, O. 1902. Eine neue Methode zur Bestimmung des Ammoniaks im Harne und anderen thierischen Flüssigkeiten. Zts. Physiol. Chem., **37**, 161–76.

Friedemann, T. E. and Graeser, J. B. 1933. The determination of lactic acid. J. Biol. Chem., **100**, 291–308.

Fromageot, Claude and Desnuelle, Pierre. 1935. Eine neue Methode zur Bestimmung der Brenztraubensäure. Biochem. Zts., **279**, 174–83.

Gillespie, L. J. and Walters, E. H. 1917. The possibilities and limitations of the Duclaux method for the estimation of volatile acids. J. Am. Chem. Soc., **39**, 2027–55.

Goodwin, L. F. 1920. The analysis of acetone by Messinger's method. J. Am. Chem. Soc., **42**, 39–45.

Gözsy, B. 1935. Mikrobernsteinsäurebestimmung und ihre Anwendung. Zts. Physiol. Chem., **236**, 54–8.

Green, D. E. 1936. α-Glycerophosphate dehydrogenase. Biochem. J., **30**, 629–44.

Hansen, P. A. 1930. The detection of ammonia production by bacteria in agar slants. J. Bact., **19**, 223–9.

Hewitt, L. 1932. Bacterial metabolism. I. Lactic acid production by hemolytic streptococci. Biochem. J., **26**, 208–17.

Hopkins, F. G. and Dixon, M. 1922. On glutathione. II. A thermostabile oxidation-reduction system. J. Biol. Chem., **54**, 527–63.

Hucker, G. J. and Wall, W. A. 1922. The use of agar slants in detecting ammonia production by bacteria. J. Bact., **7**, 513–6.

JOHNSON, M. J. 1932 Determination of small amounts of ethyl and butyl alcohol. Ind. Eng. Chem., Anal. Ed., **4**, 20–22.

JOHNSON, M. J., PETERSON, W. H. and FRED, E. B. 1931. Oxidation and reduction relations between substrate and products in the acetone-butyl alcohol fermentation. J. Biol. Chem., **91**, 569–91.

KLUYVER ET AL. 1925. Ueber die Bildung von Acetylmethylcarbinol und 2, 3-Butylenglykol im Stoffwechsel der Hefe. Bioch. Zts., **161**, 361–78.

KREBS, H. A. 1937. Role of fumarate in respiration of *B. coli commune*. Biochem. J., **31**, 2095–2124.

LANGLYKKE, A. F. and PETERSON, W. H. 1937. Determination of acetylmethylcarbinol. Ind. Eng. Chem., Anal. Ed., **9**, 163–6.

LEIFSON, EINAR. 1932. Types of bacteria on blood and chocolate agar and the immediate cause of these types. J. Bact., **24**, 473–84.

LEMOIGNE, M. 1913. Assimilation du saccharose par les bactéries du gro upe du "*B. subtilis*". Fermentation butylèneglycolique. Ann. Inst. Past., **27**,856–85.

MOYLE, D. M. 1924. A quantitative study of succinic acid in muscle. Biochem. J., **18**, 351–64.

MCCULLOCH, ANDREW. 1938. Gas Analysis, 166 pp. Witherby, London.

OSBURN, O. L. and WERKMAN, C. H. 1931. Determination of organic acids. V. Application of the partition method to quantitative determinations of acetic, propionic and butyric acids in mixtures. Ind. Eng. Chem., Anal. Ed., **3**, 264.

OSBURN, O. L., WOOD, H. G. and WERKMAN, C. H. 1933. Determination of formic, acetic and propionic acids in a mixture. Ind. Eng. Chem., Anal. Ed., **5**, 247–50.

OSBURN, O. L., WOOD, H. G. and WERKMAN, C. H. 1936. The determination of volatile fatty acids by the partition method. Ind. Eng. Chem., Anal. Ed., **8**. 270–5.

PETERS, J. P. and VAN SLYKE, D. D. Quantitative Clinical Chemistry. Baltimore, Md. 1931. Vol. **1**. Interpretations.
1932. Vol. **2**. Methods.

POTTER, V. R. and ELVEHJEM, C. A. 1936. A modified method for study of tissue oxidations. J. Biol. Chem., **114**, 495–504.

SHAFFER, P. A. and HARTMANN, A. F. 1921. The iodometric determination of copper and its use in sugar analysis. II. Methods for the determination of reducing sugars in blood, urine, milk and other solutions. J. Biol. Chem., **45**, 365–90.

SHWACHMAN, H., HELLERMAN, L., and COHEN, B. 1934. On the reversible inactivation of pneumococcal hemolysin. J. Biol. Chem., **107**, 257–65.

SIMON, L. J. and PIAUX, L. 1924. Detection and estimation of small amounts of pyruvic acid. Bull. Soc. Chem. Biol., **6**, 477–87.

SOULE, M. H. 1928. Gas Metabolism of Bacteria. *Chap. xviii in* Jordan and Falk's Newer Knowledge of Bacteriology and Immunology. Univ. of Chicago Press.

STAHLY, G. L., OSBURN, O. L. and WERKMAN, C. H. 1934. Quantitative determination of acetone and ethyl, butyl and iso-propyl alcohols in fermentation liquors. The Analyst, **59**, 319–25.

STAHLY, G. L. and WERKMAN, C. H. 1936. Determination acetylmethylcarbinol in fermentation liquors. Iowa State Col. J. Sci., **10**, 205–11.

STILES, H. R., PETERSON, W. H. and FRED, E. B. 1926. A rapid method for the determination of sugar in bacterial cultures. J. Bact., **12**, 427–39.

STRAUB, F. B. 1936. Bestimmung der Brenztraubensäure mit Salicylaldehyd. Zts. Physiol. Chem., **244**, 117–9.

THUNBERG, T. 1933. Zur Kenntnis der Spezifität der Dehydrogenasen. Biochem. Zts 258, 48–64.

VAN SLYKE, D D. 1913. The quantitative determination of aliphatic amino groups. J. Biol. Chem., 12, 275–84.

VAN SLYKE D. D. 1929. Manometric determination of primary amino nitrogen and its application to blood analysis. J. Biol. Chem., 83, 425–47.

VAN SLYKE, D. D. and CULLEN, G. E. 1916. The determination of urea by the urease method. J. Biol. Chem., 24, 117–22.

WEIL-MALHERBE, H. 1937. Studies on brain metabolism. II. Formation of succinic acid. Biochem. J., 31, 299–312.

WERKMAN, C. H. and WOOD, H. G. 1940. Gewinnung freigelöster Enzyme Spezialmethoden für Bacterien. From: Bamann and Myrbäck, Die Methoden der Fermentforschung. Geo. Thieme, Leipzig. pp. 1191–1214.

WIGGERT, W. P., SILVERMAN, M., UTTER, M. E. and WERKMAN, C. H. 1940. Preparation of an active juice from bacteria. Ia. State Col. J. of Sci., 14, 179–86.

VIRTANEN, A. I. and PULKKI, L. 1928. The volatility with steam of water soluble organic substances. J. Am. Chem. Soc., 50, 3138–51.

WENDEL, W. B. 1932. The determination of pyruvic acid and the preparation of lithium pyruvate. J. Biol. Chem., 94, 717–25.

YUDKIN, JOHN. 1933. The dehydrogenases of *Bacterium coli*. I. The effect of dilution: with a note on the existence of a co-enzyme of glucose dehydrogenase. Biochem. J., 27, 1849–58.

ZOBELL, C. E. 1932. Factors influencing the reduction of nitrates and nitrites by bacteria in semisolid media. J. Bact., 24, 273–81.

LEAFLET VII

DETERMINATION OF THE PATHOGENICITY OF AEROBES

Revised by

GEORGE H. CHAPMAN

Committeeman on Pathologic Methods

5th EDITION

Pure Culture Study of Bacteria. Vol. 16, No. 1-2

MARCH, 1948

General

Koch's postulates. Koch's postulates constitute the accepted standard for demonstrating the relation of a microorganism to disease. They are: (1) the organism must always be present where the disease occurs; (2) the organism must be obtained in pure culture from pathological tissue; (3) this pure culture must cause the disease when injected into a favorable region or tissue of a normal susceptible animal; and (4) the organism must be recovered from the latter.

Rivers (1937) pointed out that strict adherence to Koch's postulates may hinder the study of pathogenicity, particularly with regard to viruses and to the synergistic effect of two organisms. He stated that error may result even when Koch's postulates apparently have been fulfilled and that fulfillment is not always essential or desirable.

Koch's postulates are inapplicable to certain microorganisms for the reasons mentioned in the introduction and because it may be difficult to establish the presence of the pathogen in the diseased tissue even though it may be present in large numbers. (See, e.g., Chapman, 1945). In these instances, strong circumstantial evidence may be presented as "proof" of pathogenicity. However, extreme caution must be observed in drawing any such conclusions for the following reasons: about 15% of animals purchased through usual channels die from causes unconnected with the injections (Chapman, unpublished studies); infections are common in laboratory animals (see, e.g., Farris *et al*, 1945); the pathologic effects may be caused by some ingredient of the culture medium (Rigdon, 1938) and pathogenicity for one animal may not be comparable with pathogenicity for another.

Bacterial pathogenicity. Every organism that lives the normal length of life for the species passes through a life cycle. There is considerable difference of opinion among bacteriologists concerning physiological and pathogenic relationships to different stages of growth but regardless of what one considers a "life cycle" and whether "microbic dissociation" and filterable forms are included in it, there is evidence that in some pathogenic bacteria at least, differences in the growth phase are associated with differences in pathogenicity. (See, e.g., Dubos, 1945).

The pathogenicity of a microörganism can be maintained by growth in a suitable medium, animal passage, maintenance at low oxygen tension, low temperature, frequent subculturing, and drying in animal organs. Increase in pathogenic properties by animal passage is limited by exposure and selection (Zinsser and Wilson, 1932).

Different pathogenic properties. "Virulence" is used loosely to signify pathogenicity but, since different types of pathological effects are caused by different agents it is desirable to use more precise terms which designate, for example, the specific toxic power, ability to multiply rapidly in the body, etc.

Bacterial toxins. It should be determined whether the pathogenic principle is associated with intra- or extra-cellular products or is intimately connected with the bacterial cell wall. Injection of filtrates differentiates the first two from the last. Many factors, such

as peculiarities of the organism, the cultural conditions, the age of the culture and the nature of the filtering agent must be considered when testing the toxic properties of bacterial filtrates. As a rule, exotoxins are heat labile and deteriorate on standing. Scarlet fever is the most heat stable of the exotoxins and approaches the endotoxins in this respect. Heat stability may assist in differentiating the two types, but the final criterion of a true exotoxin is its ability to stimulate the production of a specific antitoxin when injected into a suitable animal. The exotoxin in a filtrate may be neutralized by the addition of immune serum and any residual toxic action may then be assumed to be due to other toxic principles. The different organs affected and the type of tissue damage should be recorded.

The Use of Laboratory Animals

For a general discussion of the care and use of laboratory animals see, e.g., Meyer (1932), Farris *et al* (1945), Cumming (1947) and Wadsworth (1947). Animals are necessary, not only for determining the etiology of specific infectious diseases and the pathogenicity of particular cultures of bacteria, but they are also utilized as a means of isolation, to determine specific pathogenic properties, to maintain organisms that grow only *in vivo*, to increase pathogenicity and to produce antibodies and other agents used in the growth and identification of microorganisms and in the diagnosis and therapy of disease.

The choice of an experimental animal and the method of injection and recovery of the organism depend upon the bacterial species and the property to be studied. The human animal would be most satisfactory in dealing with diseases of man but he is not available except on rare occasions. This limits the application of Koch's postulates in the case of man, but natural infections and accidental infection of laboratory workers are useful in supplying circumstantial evidence as to the pathogenicity of certain bacteria for man.

Healthy, previously unused animals should be employed. Several days of observation prior to injection are necessary to insure that the animals are in good condition and to provide a period of acclimatization. Following injection the animals should be observed daily for gross abnormalities and symptoms of disease and in certain cases it may be necessary to take daily temperature, pulse, respiration changes, hematology, etc. Large animals may be marked with metal tags in the ears, and the ears of small animals may be tattooed or marked with an indelible pencil.

Pathogenic bacteria produce different types of lesions in animals which may be specific and equally as important as immunological, serological and biochemical properties. To recognize them the student should be trained in pathologic technic and should be familiar with the gross and microscopic appearance of normal and diseased tissue.

Methods of Injection

Bacteria or their products which cause disease when injected parenterally may fail to do so when placed on the skin or when introduced by insufflation or by mouth. Hence the importance of different routes of injection.

The required amount of material is drawn into a sterile syringe; with the needle held up, air and any excess material is expelled onto cotton moistened with a suitable disinfectant, which should be kept away from the tip of the needle. Any undesirable disinfectant may be removed with cotton moistened with alcohol. The following types of injection are used:—

Cutaneous. This is a rather loose term and includes rubbing into, or scratching the skin or placing the inoculum under an adhesive patch. The precise method is determined by the object to be attained. If it is desired to determine whether an organism can penetrate the normal skin, the material should be spread over the skin. Irritation from shaving or depilation should be avoided. The skin should be cleansed and sterilized with an antiseptic that has brief action. The inoculated area may be covered with sterile gauze provided the adhesive does not affect the skin. Coating the skin with collodion excludes air and may make the conditions abnormal and affect the skin-penetrating power of the organism. It is common practice in cutaneous inoculation to abrade the epidermis by scratching or scraping with a sharp instrument. This aids penetration by removing the outer defensive layer and is similar to intracutaneous injection.

Intracutaneous. By intracutaneous injection is meant the introduction of material between the intradermal layers. The formation of a bleb indicates successful injection. It is advisable to use animals with unpigmented skin and rabbits should not be in moult. A 27-gage needle is best. Shaving and the application of antiseptics, particularly those that penetrate the skin, may interfere with the test and should be used judiciously.

Subcutaneous. The skin may be shaved or the hair clipped without interfering with the test. The point of puncture before injection and the puncture after inoculation should be disinfected with a non-irritating disinfectant such as tincture of zephiran chloride, alcohol, merthiolate or, best of all, green soap and water. The area may be marked with an indelible pencil. Material should be injected into the subcutaneous tissue, with care not to puncture the peritoneal wall when done in the abdomen.

If the material will not pass through the needle, the skin may be sterilized, after removing hair, and a V-shaped opening cut in the skin with sterile scissors. The flap is then lifted up and loosened until a pocket is formed and the material to be tested is inserted. The flap is replaced, sterilized and covered with collodion, or sutured aseptically.

Intramuscular. The skin is treated as for subcutaneous injection and the culture injected deep into the muscles.

Intravenous. The choice of a vein is mainly a matter of convenience and varies with the experimental animal. Rabbits usually are injected in the marginal ear vein, mice and rats in the tail veins, guinea pigs in the ear vein or jugular vein, horses and cows in the jugular vein, swine in the ear, dogs and cats in the jugular or the vein crossing the inner surface of the thigh and fowl in the radial vein that

crosses the elbow joint. If the material is considerably acid or alkaline it is adjusted to pH 7.3. The coarse particles are removed. Veins may be enlarged by rubbing with xylene or immersing them in warm water; but xylene should be avoided if the resulting reaction interferes with the test. They are washed off with alcohol before and after the injection. $HgCl_2$ should be used when working with highly pathogenic cultures. The previously warmed material free from air bubbles is slowly injected. Alcohol saturated cotton is then pressed over the puncture until the bleeding stops.

Intraperitoneal. The disinfectant is applied as with subcutaneous injection. The needle is passed through the skin and then through the abdominal wall with a short stab. *Caution:* Avoid puncturing the intestines and liver, the latter by injecting in a lower quadrant.

Intrapleural. The procedure is the same as with intraperitoneal injection except that one injects into the pleural cavity anterior to the diaphragm, the point depending upon the experimental animal. *Caution:* Avoid puncturing the lungs and pericardial sac.

Per os. Introduction of the material into the stomach or intestines may be accomplished by a catheter or capsules or by mixing the material with food or drink. To avoid exposure to the acid of the stomach the material may be enclosed in enteric coated capsules. Liquids may be mixed with starch and made into pills which are digested in the intestines. Peristalsis can be controlled with morphine.

Per Rectum.

Inhalation. Material for inhalation should be atomized in a closed space about the head of the animal. (See Rosebury, 1947, for complete details of inhalation technics).

Insufflation. Light anaesthesia is necessary to quiet the animal for insufflation. The material is blown into the trachea or bronchial tubes through a tube introduced into the larynx. Liquid may be passed into the trachea and then blown into the bronchia. In some instances the material is dropped into the nostrils and the animal is allowed to insufflate, or the material is sprayed onto the membranes of the nose and throat. The use of force and anaesthesia may reduce the resistance of the membranes. The results obtained vary with the method used, which should be reported in detail.

Intratracheal. Material may be introduced into the trachea through a tube introduced into the larynx or by means of a syringe through the side of the neck. In the latter method the skin may be incised after shaving and sterilizing it.

Ophthalmic. Material is dropped into one eye, the other serving as a control. It may also be inoculated upon the scarified bulbar conjunctiva or injected subconjunctivally.

Intracranial. Injections are made into the brain through the skull.

Intracerebral. The method varies with different species of animals depending on the material and the desired location for the inoculum. In most instances the material is deposited into one of the frontal lobes. *Caution:* Do not use enough to cause pressure. For large

animals (large rabbits and monkeys) use about 0.5 to 1.0 ml.; for medium size animals (e.g., guinea pigs) use 0.1 to 0.25 ml.; and for small animals, such as mice, use 0.02 to 0.03 ml.

Cisternal puncture. The skin is shaved and sterilized at the base of the skull over the cisterna magna. Withdraw as much fluid as is to be injected and then introduce the material with a syringe and needle, taking care not to injure nerve tissue.

Intraspinal. Injection is made between the lumbar vertebrae into the spinal canal after withdrawing an equivalent amount of fluid.

Intracardial. Attempts to inject intracardially frequently fail and numerous attempts are inadvisable. The animal should be anaesthetized, the hair clipped over the cardiac region, the skin shaved and disinfected. One should palpate for the point of maximum pulsation, insert a sharp needle (the size depending upon the animal) and feel again for the heart with the needle. When it is located, the heart beat will pulsate the needle and syringe. The needle can be easily plunged into the heart. Its entrance will be indicated by the appearance of blood in the syringe. The material must be injected slowly.

Recovery of Organisms from Blood Culture

The following factors affect the accuracy of blood cultures:—

Bacteremia. The isolation of bacteria from the blood of apparently normal animals is not related to sepsis nor to the pathogenicity of the organism. In localized infection, showers of organisms may be thrown into the blood stream at irregular intervals, necessitating repeated cultures to demonstrate them. The sequence of organisms in the blood is related to the stage of the infection, the rise in temperature and the ingestion of food. In typhoid fever the organisms are more likely to be present in the blood during the first week or 10 days, but in many other infections the best time is during the rise in fever. Organisms invade the blood stream when resistance is low.

Contamination. It is particularly important to prevent contamination when studying diseases of unknown etiology. The Keidel blood culture tube is of distinct advantage although Feder (1937) recommended a blood culture technic which he claimed had advantages over the Keidel tube. A special apparatus with the advantages of the Keidel tube and with facilities for subculturing was described by McLeod and Bevan-Brown (1918). Recently, a "Vacutainer"* appeared on the market.

The skin should be shaved and treated with a disinfectant such as mentioned for subcutaneous injections. The particular culture medium depends on the organisms expected. The antibacterial action of complement can be overcome by using the culture medium of Kracke and Teasley (1930) (See Leaflet II, p. II44–10) or by using a large volume of culture medium such as 200 ml. of broth. The smaller the amount of blood used the less chance of non-pathogenic

*Becton, Dickinson & Co.

organisms contaminating the cultures (Reith, 1926). Elliott (1938) claimed that lysis of erythrocytes and leucocytes by saponin increases the number of positive cultures when few bacteria are present.

The presence and type of antibodies for the organism recovered should be determined, particularly if the animal does not die. Recovery without the development of antibodies suggests that the organism recovered may not have been the cause of the infection but may have been a temporary invader which disappeared without stimulating much antibody production. If the animal dies, antibodies probably will not be present to any extent but if at all will be most intense just before death. Therefore, blood should be drawn immediately after death. Antibodies do not indicate pathogenicity, but they are supporting evidence.

Autopsy

The following should be determined at autopsy The cause of death; the type and distribution of the lesions; any cellular changes; distribution of the infecting organism; changes that may have taken place in the microorganism; and whether antibodies are present.

Natural infection may interfere with animal experimentation; hence, the autopsy should be made immediately after death to reduce terminal invasion. If the autopsy cannot be made promptly the body should be kept in the refrigerator. The autopsy should be done in a good light with instruments that have been sterilized by dry heat or in the autoclave.

The animal should be prepared by wetting the hair with a disinfectant that penetrates to the skin. Wetting with alcohol first helps.

Examine the area of the injection. Open the animal down the median ventral line and pull the skin back. Cover all but the exposed area with towels moistened with the antiseptic. Search for gross lesions, remove suspicious glands, tissues, etc. and place them in Petri dishes for culture and histologic examination. Moisten the exposed surfaces with alcohol and ignite.

Open the pleural cavity with a fresh set of instruments, taking care not to cut the diaphragm or pierce the lungs. If desired, seal a sample of the pleural fluid in a capillary tube and store it in the refrigerator for cytological and cultural study. Make smears and cultures of exudates. If the animal died from an infection, the organism will be abundant in most of the body fluids, and a small amount, such as a loopful, of each will lessen the chance of recovering contaminants.

Open the pericardium and sear the surface of the heart. Make an incision with a sterile instrument and proceed as with the pleural exudate.

The lungs may then be examined and any cultures or sections made. Peripheral blood may be compared with the heart blood. The blood and other body fluids may be tested for antibodies, but if the infection was of short duration they may not be detected. A high titer of antibodies for the organisms recovered suggests that they may not have caused death but this is not necessarily so because in diphtheria, e.g., the appearance of antibodies may be followed by improvement and yet the animal may die from liberated cardiotoxins.

Open the peritoneal cavity with a new set of instruments. Treat the peritoneal exudate like the pleural exudate. Sear the surface of the liver, spleen, kidney, etc. for cultures and store pieces for pathological study where indicated.

Examine all the organs, joints and cavities and make cultures where indicated. In cultures of the brain take samples from different regions to determine the distribution.

Smears made at autopsy should be stained for Gram reaction, capsules and spores.

Factors Interfering with the Determination of Pathogenicity

Factors interfering with the determination of pathogenicity were described by Teale (1933). Unless they are taken into consideration, they may lead to erroneous conclusions. An organism or its products may affect only one part of the body, and this in a specific manner, while other organisms may attack any part of the body and produce a variety of disease conditions. Different organisms may attack the same part and produce similar changes.

A pathological change in the animal tissues produced by the injection of an organism or its products indicates pathogenicity but controls must be used to exclude other factors. The ability to grow in or upon animal tissues or fluids is not of itself evidence of pathogenicity. Finally, non-pathogenic organisms may produce serological and other changes.

Variations in the resistance of individual animals or strains must also be taken into account. (As by Cumming, 1943). Infection may occur when an individual of low resistance is injected even with a normally non-pathogenic strain. Hence, several animals should always be used in tests of pathogenicity.

The following factors also interfere with the determination of pathogenicity:

Variations in the bacterial mass. Bacterial cells, like other biological units, vary around a mean because the transmission of different characters is imperfect. To reduce errors from this source it is desirable to use a culture prepared from several colonies. The cells vary with age, both naturally and in response to the environment, the latter as temporary adaptations or non-adaptative changes which may be transmitted through successive generations and then disappear. The changes rarely result in mutations. Holman and Carson (1935) discussed precautions that must be observed in the study of bacterial variation.

Natural variations. Natural or normal variations include variations of individual cells around the mean and variations resulting from the life cycle which may vary in all the morphological, physiological and pathogenic properties of the culture. Selective cultivation and animal passage of cultures that have lost pathogenicity may lead to development of pathogenic cells in the culture. Some non-pathogenic cultures may contain pathogenic variants, particularly if the culture was associated with a disease process. Hence, the advisability of testing a number of colonies separately.

If, as some still believe, the normal life cycle of a bacterial cell consists mainly of an increase in size with age, with minor morphological and physiological changes, the relationship of pathogenicity to the life cycle has little significance. If, however, the life cycle is represented by complicated ontogenetic changes (dissociations), each phase or stage representing distinct characteristics and varying in stability and in response to the environment, the relationship of pathogenicity assumes considerable importance. This problem concerns Leaflet VII only in so far as the variations affect the study of pathogenicity.

As regards pathogenicity, dissociation may occur as readily in this property as do morphologic and physiological changes in the cell or colony and may be associated with one or more of these latter changes. The relationship of pathogenicity to R, S, M and G colony types and to the morphology, size and age of the cell may have to be determined for each culture. With some organisms, e.g., *Salmonella typhosa* and *Corynebacterium diphtheriae*, the smooth colony type is the most pathogenic, whereas the mucoid phase of *Diplococcus pneumoniae* and *Klebsiella pneumoniae* and the rough phase of other organisms, such as *Bacillus anthracis*, are the most pathogenic. The relation of the G phase to pathogenicity has not been clearly established but in staphylococci, e.g., it appears to be non-pathogenic.

Acquired variations. Acquired or new variation represents changes in the average cell in response to environmental changes. When they are favorable to survival of the organism they are adaptations.

Organisms not ordinarily pathogenic may acquire some degree of pathogenicity in animal passage but they are not strictly pathogenic. Consequently, the history of an organism *in vivo* should always be reported. Organisms grown in immune serum may increase in pathogenicity and resist agglutinating and other antibodies.

Some organisms lose pathogenicity quickly, particularly when grown on artificial culture media. They are usually most pathogenic in the late logarithmic phase. To reduce this tendency to lose pathogenicity the culture medium and incubation temperature should favor optimum growth and should be similar to conditions existing in body fluids and tissues (See Felton, 1932). Tissue culture or fresh blood, either unheated or inactivated at 57°C for 1 hour to destroy transient organisms, used alone or added to the culture medium are valuable in maintaining pathogenicity. Transferring from one animal to another should be done quickly.

Antigenic variations. In addition to those changes in antigenic specificity associated with different phases, there is some evidence that bacteria may adsorb antigen from the environment with resulting change in antigenicity (See, e.g., Burky, 1934 and Rosenow, 1945). False serologic reactions have resulted from foreign antigen, such as agar. Two different organisms may have a common antigen from being grown on the same medium. Thus, a common antigen may not necessarily indicate a natural relationship (See, e.g., Dubos, 1945). Extraneous or unnatural antigens or their antibodies

may sometimes be eliminated by growing the organisms on different culture media. This would be simpler than by adsorbing immune sera with a common antigen.

Distinction should be made between adsorption of a foreign antigen, change in the bacterial antigen and physical mixture of a foreign antigen. The latter can be removed by thorough washing with saline. In working with obligate parasites, particularly filterable forms, the difficulties are increased due to antigens present in tissues.

The occurrence of heterophile or non-specific antigens and antibodies complicates the study of pathogens. Yeast and *Klebsiella pneumoniae* stimulate immunity to Type II pneumococci, injection of sheep cells produces immunity to anthrax (Rockwell, 1933) and injection of *Salmonella pullorum* stimulates antibodies against *S. schottmulleri*, *S. paratyphi*, *S. (Eberthella) typhosa* and *Shigella dysenteriae*. Therefore, the presence in the blood of antibodies for a particular organism is not of itself convincing evidence that the organism caused the infection or that it acted as the antigen.

The number, nature and natural occurrence of non-specific antigens, their relationship to phase variations and their distribution should be determined. Methods for studying non-specific antigens concern Leaflet VIII.

Obligate parasites. Still greater difficulties are encountered in determining the pathogenicity of obligate parasites. The direct transfer of body fluids or tissues involves the objections just discussed. Also, two organisms or non-specific antigens may be present, as in typhus fever, and the immune sera produced when the animal tissues or fluids are used as antigens may contain antibodies for both organisms, making serological evidence inconclusive.

The presence of organisms in tissue or in the blood stream is not necessarily evidence of pathogenicity or parasitism. Organisms from different sources are continually entering the blood stream and dead tissues may be present in living animals permitting non-pathogenic saprophytes to flourish. For these reasons, the only indication of pathogenicity in the case of obligate parasites consists of an accumulation of circumstantial evidence. Improvements in tissue culture technic may provide a solution.

Specificity. The pathogenicity of an organism may be confined to a single species of animals, which stresses the importance of the proper selection of an experimental animal. The designation of an organism as pathogenic or non-pathogenic, etc. refers solely to the animal and method used.

Passage through one animal may result in reduced pathogenicity for another. The pathogenicity of an organism for a different species of animal should be tested with cultures grown on artificial culture media for some time as well as with freshly isolated cultures.

Synergism. Occasionally two organisms may grow together and produce a pathologic condition whereas neither can do so alone. Both organisms do not necessarily produce toxins and it is possible that one of them may in no way contribute directly to the disease

even though it may be universally present. One may be a harmless invader, constantly associated with the disease but not contributing to it. Even when both organisms are essential to produce the disease, one may be a saprophyte in dead tissues and may contribute to the infectious process only by providing conditions essential to the growth of the pathogen. Unless the organisms are also associated in other diseases, serologic tests may be of differential value. Bacterial antagonism also plays a role in pathogenicity in some instances.

It should be determined whether the bacterial product causes disease by its direct action on the tissues or by sensitizing them to it. If the latter, then other organisms that produce a similar antigen or a similar non-specific antigen may also account for the pathology. A pathogenic organism can be differentiated because it grows in the animal and produces sensitization (Hanger, 1928).

Cultural considerations. The cultivation of pathogenic bacteria may not always be favorable for producing the pathogenic factors. *Corynebacterium diphtheriae* and streptococci, e.g., grow luxuriantly under certain conditions without producing toxin. Certain bacteria require oxygen for toxin production. The toxin also may be produced and then disappear in a culture or may be destroyed by unfavorable manipulation. Finally, an early toxin and a late one may have different properties.

Most pathogenic aerobes are facultative anaerobes or facultative microaerophiles. Parasitic species may prefer tissues or cavities with low oxygen tension.

The Use of Biochemical Methods in Lieu of Animal Inoculation Tests to Study Certain Pathogenic Properties

Because they give results parallel with certain pathogenic effects, tests have been proposed, based on biochemical properties, that appear to be satisfactory as substitutes for animal inoculation experiments, e.g., when a large number of cultures are to be tested as in clinical work, when animal inoculation experiments are inconclusive, as in non-hemolytic streptococci, or when animal inoculation experiments involve considerable danger (see, e.g., Dozois and Rauss, 1935; and De Angelis, 1937). For example, power to clot plasma is now recognized as an excellent *in vitro* method for differentiating pathogenic from non-pathogenic staphylococci. For a summary of recent biochemical methods for staphylococci, see Chapman (1946).

Resistance of streptococci to the bactericidal power of fresh, diluted, defibrinated guinea pig blood and to different chemicals is an excellent indicator of pathogenicity (probably toxicity). A complete up-to-date discussion of this work will be found in Chapman (1947).

Although the writer is enthusiastic about carefully applied biochemical tests of such organisms as staphylococci and streptococci as substitutes for animal inoculation tests, he is aware of their shortcomings and is not in favor of universal acceptance at the present time. So many technical considerations enter into the reliability

of the methods that few bacteriologists possess the technical knowledge or skill to apply them satisfactorily.

Summary

It is obvious that suitable technic, skilfully applied, and extreme caution in interpretation of the results are necessary to determine the pathogenic properties of microörganisms. Indirect evidence is acceptable as a substitute when Koch's postulates are inapplicable or when animal inoculation experiments are not entirely satisfactory; but such evidence is rarely sufficiently conclusive except as a working hypothesis. In the absence of conclusive evidence, the organism should be considered pathogenic only so far as the experiments indicate.

To facilitate study of an investigation by others the methods used should be reported punctiliously.

REFERENCES

Burky, Earl L. 1934. Production of lens sensitivity in rabbits by the action of staphylococcus toxin. Proc. Soc. Exp. Biol. & Med., **31**, 445.

Chapman, George H. 1945. Staphylococci in gastroenterology. Am. J. Digestive Dis., **12**, 399.

Chapman, George H. 1946. The staphylococci. Trans. N. Y. Acad. Sci., **9**, 52.

Chapman, George H. 1947. Relationships of nonhemolytic and viridans streptococci in man. Trans. N. Y. Acad. Sci., **10**, 45.

Cumming, C. N. Wentworth. 1943. The importance of *Mus musculus* in research: A discussion of its genetic aspects. Presented before the Am. Public Health Assn., New York, Oct. 11–14.

Cumming, C. N. Wentworth. 1947. Modern mass production in animal breeding for experimental research. *In press.* Read before the Fourth International Cancer Research Congress.

De Angelis, Eugene. 1937. A reaction with iron compounds for the determination of *B. anthracis* and of its pathogenicity. J. Bact., **33**, 197.

Dozois, K. Pierre and Rauss, K. F. 1935. Relationship between electrophoretic migration velocities, the virulence and the types of the diphtheria and diphtheria-like bacilli. Am. J. Pub. Health, **25**, 1099.

Dubos, René J. 1945. The Bacterial Cell In Its Relation to Problems of Virulence, Immunity and Chemotherapy. Harvard Univ. Press.

Elliott, S. D. 1938. The use of saponin in blood culture media, with special reference to blood cultures in subacute bacterial endocarditis. J. Path. & Bact., **46**, 121.

Farris, Edmond J., Carnochan, F. G., Cumming, C. N. W., Farber, Sidney, Hartman, Carl G., Hutt, Frederick B., Loosli, J. K., Mills, Clarence A. and Ratcliffe, Herbert L. 1945. Animal colony maintenance. Ann. New York Acad. Sci., **46**, (Art. 1) 1.

Feder, F. M. 1937. A new and simplified blood culture technic. J. Lab. & Clin. Med., **22**, 846.

Felton, Lloyd D. 1932. Studies on Virulence: Influence on virulence of pneumococci of growth on various media. J. Exp. Med., **56**, 13.

Fulton, F. 1943. Staphylococcal enterotoxin—with special reference to the kitten test. Brit. J. Exp. Path., **24**, 65.

Hanger, Jr., Franklin M. 1928. Effect of intravenous bacterial filtrates on skin tests and local infections. Proc. Soc. Exp. Biol. & Med., **25**, 775.

Holman, W. L. and Carson, Arline E. 1935. Technical errors in studies of bacterial variations. J. Inf. Dis., **56**, 165.

Kracke, Roy R. and Teasley, Harry E. 1930. The efficiency of blood cultures. J. Lab. & Clin. Med., **16**, 169.

McLeod, J. W. and Bevan-Brown, R. E. 1918. The technique of blood culture. J. Path. & Bact., **22**, 74.

MEYER, K. F. 1932. Use of animals in routine diagnostic work. J. Lab. & Clin. Med., **17**, 510.

REITH, ALLAN F. 1926. Bacteria in the muscular tissues and blood of apparently normal animals. J. Bact., **12**, 367.

RIGDON, R. H. 1938. Observations on Dolman's test for determining the presence of staphylococcal enterotoxin. Proc. Soc. Exp. Biol. & Med., **38**, 82.

RIVERS, THOMAS M. 1937. Viruses and Koch's postulates. J. Bact., **33**, 1.

ROCKWELL, GEORGE E. 1933. Active immunization to anthrax by means of heterophile antigen. Science, **77**, 612.

ROSEBURY, THEODOR. 1947. Experimental Air-Borne Infection. Williams and Wilkins, Baltimore, Md.

ROSENOW, EDWARD C. 1945. Production in vitro of substances resembling antibodies from bacteria. J. Inf. Dis., **76**, 163.

TEALE, F. H. 1933. Factors influencing the pathogenicity of bacteria. J. Path. & Bact., **37**, 185.

WADSWORTH, A. B. 1947. Standard methods of the Division of Laboratories and Research of the New York State Department of Health. Williams and Wilkins, Baltimore, Md.

ZINSSER, HANS and WILSON, E. B. 1932. Bacterial dissociation and a theory of the rise and decline of epidemic waves. J. Prev. Med., **6**, 497.

LEAFLET VIII

SEROLOGICAL METHODS

6TH EDITION
Pure Culture Study of Bacteria, Vol. 15, No. 3-4
NOVEMBER, 1947

Revised by
C. A. STUART and K. M. WHEELER

LEAFLET VIII

SEROLOGICAL METHODS

THE USE OF SEROLOGY IN PURE CULTURE STUDY

In the study of bacteria the facts that may be established with the agency of serological reactions often have peculiar value, not as substitutes for those to be gained from morphological, cultural, or biochemical means, but as supplemental to them. This is especially true in so-called "pure culture" investigations. Thus, serological studies may show that a group of organisms, apparently alike morphologically and physiologically, in reality consists of different sub-groups, which cannot be distinguished by other means. This leaflet is intended to make readily available such serological procedures as are useful in pure culture studies. No implication is made that these procedures are necessarily the best among the great variety of serological methods now in use. They have, however, proved practical in pure culture studies, and they are given herewith the hope that they will be helpful to users of this Manual.

The field designated "serology," as applied to pure culture study of bacteria, deals with the reactions of the blood sera of animals that have been injected with micro-organisms or their products. Such substances, acting as antigens when injected into an animal, stimulate the appearance of antibodies in its blood serum. This serum when mixed with suspensions of unknown bacteria or their products, gives a positive reaction only if the bacteria or their products are of the same type as those introduced or else are related to them. This specificity is not absolute but may vary within certain limits both quantitatively and qualitatively. It is often possible, however, by means of these relatively specific reactions to identify an unknown organism, to group or sub-group closely allied forms, and to study the relationship between the groups, sub-groups, and strains. Divisions so established may or may not agree with previous groupings based upon morphological, cultural or biochemical observations. As a rule the methods will be found supplemental to each other and more often than not their results will coincide. Frequently, when other procedures fail to show differences, serological reactions will reveal them and varieties of a given species may be differentiated by their antigenic properties.

Immune sera can yield information in two ways: either a known serum may be employed as the test agent for determining an unknown antigen; or a known antigen may be used as a test agent to denote the presence or absence of a specific antibody. Serological procedures that may be useful in the identification of pure cultures are:

1) Agglutination: agglutinogen (antigen)-agglutinin reactions; agglutinin absorptions.
2) Precipitation: precipitinogen (antigen)-precipitin reactions.
3) Complement fixation: antigen-antibody-complement-hemolysin-RBC reactions.

4) Toxin neutralization: toxin-antitoxin reactions.

5) Hemolysis: hemolysin reactions. (See Leaflet VI, pages VI$_{42}$-16–18.)

The most frequently employed serological reactions for the identification of bacteria are those of agglutination and complement fixation. The agglutination method is especially rapid and reliable. Adequate controls on all reagents are essential for each procedure.

DEFINITION OF TERMS

An *antigen* is defined as a substance which, when introduced parenterally into an animal body, stimulates the animal to produce specific bodies that react or unite with the substance introduced. In this outline of methods the term will be limited to suspensions of living or killed bacteria or their products. *Agglutinogen*, *precipitinogen*, *toxin*, and *toxoid* are some of the names applied to antigens employed in the various serological procedures.

An *antibody* is the specific body above mentioned, produced by the animal in response to the introduction of an antigen. These antibodies under the right conditions may act as one of the principal factors in preventing any injurious action which the antigen might otherwise exert. For use in practical serology, antibodies are obtained from the blood serum and appear in the globulin fraction. *Agglutinin*, *precipitin*, and *antitoxin* are designations in common use.

Complement is a third substance which may take part in serological reactions. It is present in varying degree in the normal serum of all animals, combines with the antigen-antibody union and may bring about lysis of the bacteria, is non-specific and is not increased during immunization, and in contrast to bacterial antibodies which are relatively heat stable, is inactivated by exposure at 56°C for 30 minutes and deteriorates in a few days at refrigerator temperature.

A *hapten* or partial antigen is that portion of an antigen which contains the chemical grouping upon which the specificity depends. The hapten reacts specifically with the corresponding antibody, but by itself, when separated from the carrier molecule, is incapable of stimulating the formation of antibodies *in vivo*.

Heterophile antigen is the term applied to common antigens which may occur in the tissues of animals which are not closely related. Several have been described of which the Forssman heterophile antigen is an example. When guinea pig kidney emulsion is injected into rabbits an antibody (Forssman antibody) is formed which reacts with sheep erythrocytes. The Forssman antigen has been found in several species of bacteria. Among bacteria, common antigens may be found in groups that are widely divergent in morphological and biochemical characters.

BACTERIAL DISSOCIATION

Bacteria may produce variants which differ from the parent in one or more characters such as colony form, morphology, virulence, biochemical activity or antigenic composition. Variation in serological reactivity may or may not be correlated with other variation, may occur naturally or be induced, and may or may not be a stable change. There is, for example, change from smooth (S) to rough (R) with intermediate phases, variation in motility or presence of *flagellar* (H) antigens, form variation or change of *somatic* (O) antigen, phase variation of flagellar antigen involving change in serological reactivity of motile forms. These and other variations in the antigenic constitution of bacteria must be considered in pure culture study by serological methods, and these variations are often the explanation of anomalies observed in the results of serological tests.

AGGLUTINATION

The antibodies in the blood serum of immunized animals that cause clumping or agglutination of bacteria are called agglutinins. Agglutinins may occur naturally, but if present are usually weak.

The agglutination of a suspension of bacteria by its homologous immune serum may be observed either microscopically or macroscopi-

cally. The macroscopic tube test is probably the most accurate and is a convenient method. The macroscopic slide test has been used extensively for typing enteric bacteria. The phenomenon of agglutination is evidenced by the appearance of granulation in the bacterial suspension. Granulation may be extremely fine, or clump size may range to very coarse. Clumps may be compact and dense as in the case of somatic agglutination, may be light and cottony with flagellar reactions, or may be stringy and thread-like with some mucoid organisms. Conditions of optimal incubation time and temperature vary considerably depending on the organisms tested.

PREPARATION OF IMMUNE SERUM

The rabbit is the most satisfactory animal for the production of agglutinating serum. The technics employed for immunization are many, and vary widely with different workers. The particular properties of the bacteria under examination are the factors determining the method chosen.

A healthy, well-developed rabbit is selected for immunization. It may conveniently be held in a squatting position by an attendant or locked in a special box in such a manner that only the head protrudes. The hair is removed from around a marginal ear vein and along the edge of the ear by shaving. The ear is then cleansed with 70% ethyl alcohol. Dilation of the vein is promoted by rubbing or patting, by heat, or by applying xylol. Any sharp sterile instrument will serve to open the vein, a clean wound favoring the escape of blood. About 5 ml. of blood are collected in a sterile test tube to provide serum for determining the presence or absence of natural agglutinins in the blood of the rabbit selected. The technic is described in the next section. Having determined the absence of natural agglutinins the immunization procedure may be undertaken.

Immune Serum: Immunize the rabbit by repeated subcutaneous, intraperitoneal, or intravenous injections of saline suspensions of young (18–24 hour) cultures of the organism to be studied. Growth can be taken from agar or from silica gel medium or in the case of flagellar antigens young broth cultures may be used. A convenient suspension is one containing about 500 million organisms per milliliter, although suspensions containing more or less than this number of organisms can be used, depending upon the toxicity of the culture. The number of organisms in the suspension can be quickly and roughly determined by various methods outlined in any treatise on serological methods. If the organism is non-pathogenic for rabbits, suspensions of living organisms can be injected. As a rule, however, the organisms are killed before injection by heating the suspension in a water bath at 60° C. for 1 hour, or by mild chemical treatment such as 0.3% formalin. Inject subcutaneously, intraperitoneally, or intravenously into a rabbit at intervals of 5–7 days starting with a dose of 0.5 ml. and increasing each dose by 0.5 ml. After the third injection a test bleeding may be made to determine titer, and the rabbit bled out or reinjected as necessary. Titrations of the serum should be made following each subsequent injection, and immunization continued until a satisfactory titer is attained or until no further increase occurs. Six to eight injections are usually required to produce agglutinins of sufficient titer. The method of immunization can be varied to meet the needs of special cases. The shorter the period of immunization, the more specific is the immune serum. Long immunization increases the content of group agglutinins. To test the titer of the serum, draw 1–2 ml. of blood from the marginal vein of the ear of the rabbit 5–7 days after the last injection; collect the serum and carry out an agglutination test with it, as described below. If the titer is sufficiently high, bleed the rabbit from the heart or an artery to obtain as much blood as possible. Allow the blood to clot, collect the serum aseptically, and add 0.5% phenol or 0.3% tricresol to the serum as a preservative. Place the serum in ampules or bottles and store in the refrigerator.

Bleeding of Rabbit: Rabbits are more easily and readily bled from the heart when large amounts (25–100 ml.) of blood are desired. Etherize the rabbit, clip the hair over the region of the heart, and shave. An added precaution to prevent contamination is to wet the hair of the rabbit thoroughly over the entire left side. With the rabbit lying on its right side paint the shaved area with tincture of iodine. Determine the point of maximum pulsation. Using a sterile 50 ml. Luer syringe and a needle of 17 or 18 gauge (2–2½ inches), insert the needle at the point of maximum pulsation. The

heart can now be located with the needle which will be moved by the heart beat. Force the needle into the heart. When it is in the heart, blood will flow into the syringe. Slowly withdraw 50 ml. Quickly withdraw the needle and eject the blood into a 500 ml. Erlenmeyer flask, or into a large test tube and allow it to clot. The serum may be obtained free of clot and cells by centrifugation. Five to six pound rabbits can be bled monthly in this way. If the rabbit is to be sacrificed, another 50 ml. portion of blood can be obtained in a similar manner, but preferably with another clean sterile syringe and needle in order to avoid clotting in the syringe.

PROCEDURE

Procedure for Microscopic Agglutination Test: Dilutions of the serum are prepared by diluting the immune serum with saline solution, care being taken to keep the serum twice the strength of the final dilution desired, since the addition of an equal volume of the antigen doubles the dilution of the serum on each cover slip. Upon separate clean cover slips is placed a loop of the diluted serum. A loop of the suspension of the organism is placed beside each drop of diluted serum and the two mixed with a platinum wire. The cover slips are then suspended over hollow ground slides as noted in the technic for preparing a hanging drop preparation. The slides may be held at room temperature for a short time, usually less than one hour, and examined under a magnification of approximately 500 diameters.

Some experience is necessary to discriminate between normal reactions and false clumpings. In the true reaction all the organisms in the field will be gathered into a few clumps and no organisms will be found around the edges of the drop. In pseudo-reactions the organisms may collect around small foreign particles, around the edge of the drop, and in many small clumps containing a relatively small number of cells. The beginner generally uses too heavy suspensions. Much sharper readings can be made with a very light suspension of the organism being studied.

Macroscopic Agglutination Test. Antigen: Wash off in saline the growth from a 24-hour agar slant culture of the organism to be tested. An emulsion which is too thick obscures the agglutination, while one which is too thin does not provide enough bacteria for macroscopic comparisons. The density of the emulsion of bacteria must be adjusted to meet the requirements of special conditions and to assure constancy in the results. This adjustment can be made on the basis of an actual count of the number of bacteria per ml. or by comparison with a standardized suspension of insoluble particles. The latter method is usually more convenient, using the McFarland (1907) nephelometer. A density of 0.5 on the McFarland nephelometer scale is satisfactory for most purposes. The suspension should be homogeneous, "smooth", and entirely free from particles. The bacteria in the suspension may be killed by heat at 60°C. for 1 hour, or living bacteria may be used. Satisfactory preservatives for a suspension for the agglutination test are 0.5% phenol or 0.3% formalin.

Some suspensions of bacteria tend to flocculate spontaneously, necessitating as a control a suspension of the bacteria in saline which is carried through the incubation period of the test. Spontaneous agglutination may be due to many factors, such as surface tension, electrical charges upon the surfaces of the bacteria and other un-

known conditions associated with the composition of the bacterial cell. Spontaneous flocculation can at times be avoided by proper regard to the pH of the suspending fluid with the use of buffer mixtures, by passing the organism through several transfers immediately before the final culture to be used in making the suspensions, and by the growth of the organisms in media which favor diffuse growth. Washing the organisms in distilled water, ether, and chloroform, and taking the supernatant fluid from heavy suspensions which have been allowed to sediment are procedures which may make it possible to obtain a smooth suspension of an organism which originally flocculated spontaneously in saline.

Procedure for Macroscopic Agglutination Test: The test is performed by mixing a constant amount of the bacterial suspension (antigen) with decreasing amounts of the antiserum, according to the protocol in Table 1.

TABLE 1

COMPLETE AGGLUTINATION TEST—WITH RESULTS IN A TYPICAL INSTANCE

Tube No.	0.85% aqueous NaCl	Immune serum: preparation of dilution	Dilution	Antigen (bacterial suspension)	Final dilution	Incubation in water bath at 50–52°C. for 2–4 hours	Agglutination observed in a typical instance
1	0.9 ml.	0.1 ml. of immune serum*	1:10	0.5 ml.	1:20		complete
2	0.5 ml.	0.5 ml. from tube No. 1	1:20	0.5 ml.	1:40		complete
3	0.5 ml.	0.5 ml. from tube No. 2	1:40	0.5 ml.	1:80		complete
4	0.5 ml.	0.5 ml. from tube No. 3	1:80	0.5 ml.	1:160		complete
5	0.5 ml.	0.5 ml. from tube No. 4	1:160	0.5 ml.	1:320		complete
6	0.5 ml.	0.5 ml. from tube No. 5	1:320	0.5 ml.	1:640		complete
7	0.5 ml.	0.5 ml. from tube No. 6	1:640	0.5 ml.	1:1280		complete
8	0.5 ml.	0.5 ml. from tube No. 7	1:1280	0.5 ml.	1:2560		partial
9	0.5 ml.	0.5 ml. from tube No. 8	1:2560	0.5 ml.	1:5120		none
10	0.5 ml.	———	———	0.5 ml.	———		none

*The contents of each tube should be thoroughly mixed by sucking up the fluid in the pipette and blowing it back into the tube several times before transferring the 0.5 ml. to the next tube. After mixing, 0.5 ml. is discarded from tube No. 9. A one ml. pipette graduated to tip is the most convenient size. Tubes of about 10 mm. inside diameter are suitable for this volume of fluid.

Starting with the 1:10 dilution of this antiserum, the series of dilutions can be made readily in the same tubes in which the test is to be done. Tube 10 is used as a control for the smoothness of the bacterial suspension. It should be free from clumps. After the antigen is added, shake well and incubate for 2–4 hours at 50 to 52°C.[1] After this period of incubation readings may be taken at once, or the tubes may be allowed to stand overnight at room temperature or preferably in the refrigerator.

[1]The time and temperature of incubation is not the same for all bacteria. Agglutination proceeds more rapidly with motile than with non-motile bacteria. Agglutination of non-motile bacteria may be accelerated by shaking or by taking advantage of the convection currents set up in the tubes where the level of the water is below the level of the liquid in the tubes.

Readings and Results: At the end of the period of incubation, for the test to be satisfactory, the control tube should show a uniform cloudiness without sedimentation or flaking. A positive reaction will vary in appearance with the type of agglutination which has taken place. With progressive dilutions the reduction in the quantity of agglutinins is accompanied by less and less complete agglutination. This is observed in the tube as decreased amounts of sediment and less marked granulation or clumping. Conversely, it is associated with correspondingly increased turbidity of the supernatant fluid and closer and closer approximation to the appearance of the control tube. The titer of the agglutinin is taken as the highest dilution in which agglutination takes place. Certain immune sera agglutinate only in the higher dilutions. The failure of relatively concentrated serum to cause agglutination has been designated by such terms as "prezone," "prozone", and "zone of inhibition." *Example:* If in Table 1 (*it is to be emphasized that the results set down in this table are arbitrarily chosen to serve as an example only*) no agglutination resulted in tubes Nos. 1 to 3, partial clumping in tube No. 4, complete agglutination in tubes Nos. 5 to 7, while in the succeeding tubes the reactions were less and less complete, then a zone of inhibition would be indicated in the concentrations of the sera employed in tubes Nos. 1 to 3. When absence of clumping is seen in one or more tubes other than at the beginning of a series it is usually due to an error in technic. Zones of inhibition should always be guarded against by using a sufficient range of dilution of the antiserum, lest a false negative result appear. Great care in carrying out the steps in agglutination technics is essential if accurate results are to be obtained by such methods.

The macroscopic slide agglutination test is performed on a glass slide using a drop of serum dilution plus a drop of heavy bacterial suspension (density of McFarland 7–8). Serum and antigen are mixed over a surface of about 1 cm. diameter and mixing is continued by rocking the slide. The degree of clumping is read after about 2 minutes. While the slide technic has advantages of simplicity and speed, the macroscopic tube test provides a more reliable and adaptable technic for pure culture study.

For the complete identification of a bacterial strain, agglutination to titer should be secured with an antiserum produced with organisms of known type; and, furthermore, the organism in question, if used in sufficient quantity, should absorb all of the agglutinins from such an antiserum, thus leaving the antiserum devoid of agglutinating power against both the organism in question and the organism used to produce the antiserum. Partial agglutinin absorption may indicate a degree of relationship. In order to establish the identity of two bacterial strains complete cross-agglutination and cross-absorption should take place between the two organisms and the two antisera.

Attention may be directed here to the phenomenon of "group agglutination" which results from common agglutinins acting on bacterial species which are closely allied to each other. An example is to be found in the colon-paratyphoid-typhoid-dysentery group. The absence of exact specificity in agglutination reactions is due to the

group agglutinins. In dealing with a bacterial division such as that cited above, group agglutinogens and agglutinins are encountered in addition to strain-specific agglutinogens and agglutinins.

AGGLUTININ ABSORPTION

Agglutinin becomes attached to bacteria which are mixed with an homologous antiserum, and can be removed from the fluid by the removal of the bacteria. This is known as the absorption of agglutinin. Some inagglutinable organisms retain the capacity to link up with the antibody (agglutinin) and hence, like agglutinated bacteria, are capable of absorbing agglutinin. The absorption of agglutinins with agglutinable and inagglutinable strains of bacteria has become an extremely important serological procedure for determining identity of bacterial strains and for establishing group relationships. The scope of this MANUAL does not permit consideration of all the factors involved in this reaction nor the description of the several technical procedures which have worked well in the hands of different investigators. It is to be emphasized that highly significant results in pure culture studies can be obtained by the application of this method *after* the user has become thoroughly conversant with the technic and is familiar with the conditions which influence it.

Two principles govern the application of the test for the absorption of agglutinin. According to one principle, the ability of individual strains to absorb agglutinins from type antisera is tested. A given organism is considered to be identical with the type strain when it completely absorbs the agglutinins from the type antiserum and when the type organism completely removes the agglutinins from the antiserum for the organism being studied. According to the second principle, the agglutination of organisms by type sera from which group agglutinins have been previously removed is tested. Each method has its special advantages. The first method gives the more precise results and will be described below, as it includes the chief procedures which would be used in the application of the second method.

Procedure for Absorption of Agglutinin: At the start, the agglutinating antisera are prepared according to the method described. The antigens are prepared in the same manner as those used in the agglutination test. Dense suspensions are used for the absorption of agglutinins, while the usual type of suspension (0.5 on McFarland scale) is employed in the tests with the absorbed sera.

To prepare the absorbing antigen, wash off the bacteria from agar slants or petri dishes into a small amount of saline. Filter through absorbent cotton if necessary to obtain a smooth suspension. Absorption is accomplished by adding the concentrated antigen to serum diluted 1:20 or 1:40 and removing the bacteria by centrifugation after a period of incubation at room temperature for one half hour or at 37°C for 1 hour. The minimal absorbing dose of bacteria for a given volume of serum can be determined by varying the absorbing dose and selecting the smallest one which completely removes the agglutinins for the absorbing strain. Successive absorptions with 2 or 3 doses are more efficient in removing antibodies than a single absorption with the same total amount of bacterial suspension. In identifying unknown strains, doses 2–4 times the minimal dose are used. After absorption the serum is tested for its ability to agglutinate the homologous strain, and any other strains of bacteria used in the study. These agglutination tests are set up with dilutions covering the original serum range and extending as low as 2.5% of the original titer of the serum. It is important to cover the entire range of the titer of the serum. At times pre-zone phenomena occur which would lead to a false result if only a single dilution were used in the final test for agglutination.

PRECIPITATION

The precipitin reaction may be used in the examination and identification of bacterial extracts and autolysates. The reaction involves the mixing of antigen and antiserum, with a resultant precipitate or ring formation if the two are homologous. This is one of the most delicate serological methods. Sera may be obtained which detect the specific antigen in dilutions as high as 1:100,000. A serum which will react in dilutions of 1:10,000 or 1:20,000 is not uncommon.

PRODUCTION OF PRECIPITINS

Rabbits are suitable animals for the production of precipitins. It may be necessary to use several rabbits, since some rabbits are refractory. Precipitins for bacterial proteins may be produced in the rabbit by using as antigen bacterial suspensions, filtrates, extracts or autolysates. However, the antigen employed for the *in vitro* test must be in solution, clear, and free from antiseptics. Clarification

TABLE 2

PROTOCOLS FOR THE PRECIPITIN TEST

A. Antigen dilutions*

Tube	0.85% aq. NaCl	Antigen	Vol.	Vol. of dil'd antigen remaining after all dilns. are made	Dilution of remaining antigen
	ml.		ml.	ml.	
1	0.9	0.1 ml.	1.0	0.9	1:10
2	0.9	0.1 ml. of No. 1	1.0	0.5	1:100
3	0.5	0.5 ml. of No. 2	1.0	0.5	1:200
4	0.5	0.5 ml. of No. 3	1.0	0.5	1:400
5	0.5	0.5 ml. of No. 4	1.0	0.5	1:800
6	0.5	0.5 ml. of No. 5	1.0	0.5	1:1,600
7	0.5	0.5 ml. of No. 6	1.0	0.5	1:3,200
8	0.5	0.5 ml. of No. 7	1.0	0.5	1:6,400
9	0.5	0.5 ml. of No. 8	1.0	0.5	1:12,800
10	0.5	0.5 ml. of No. 9	1.0	0.5	1:25,600

B. Precipitin test

Tube	Precipitin serum	Antigen†		0.85% aq. NaCl	Result
		Dilution	Amount		
	ml.		ml.		
1	0.1	1:100	0.1	.....	.....
2	0.1	1:200	0.1	.....	.....
3	0.1	1:400	0.1	.....	.....
4	0.1	1:800	0.1	.....	.....
5	0.1	1:1,600	0.1	.....	.....
6	0.1	1:3,200	0.1	.....	.....
7	0.1	1:6,400	0.1	.....	.....
8	0.1	1:12,800	0.1	.....	.....
9	0.1	1:25,600	0.1	.....	.....
10	0.1	.....	..	0.1 ml.	Control tubes‡
11	..	Undiluted	0.1	0.25 ml.	

*Use ordinary size test tubes for these dilutions. Mix the contents of each tube thoroughly before transferring to another tube for further dilution.

†Using dilutions made in A (above).

‡Other controls may be added when deemed advisable.

and sterilization of the antigen may be done by filtration (Berkefeld). High titered sera have been produced by injecting progressively increasing doses of antigen at 3-day intervals. After 5–6 injections, a test bleeding is made and if the titer is low additional injections are given. Bleedings are made a week after the last injection. When a sufficiently high titer has been reached, the rabbit is bled aseptically from the heart;[1] the blood is allowed to clot; and the clear serum removed to sterile ampules which are sealed and labeled. Preservatives should not be added as they tend to interfere with the precipitin test. The serum should be perfectly clear and free from fat and hemoglobin. It should be stored at about 4°C. If necessary the serum may be filtered (Berkefeld). The titer of the precipitating serum is determined by ascertaining the highest dilution of the antigen with which the serum forms a precipitate or ring test in two hours at 37°C. (optimum temperature). The precipitate consists very largely of the globulin and lipids of the precipitating serum.

PROCEDURE

1. Progressively doubled serial dilutions of antigen are prepared in saline beginning with 1:100. (Table 2).
2. One-tenth ml. of the serum is transferred to the bottom of small tubes (5×50 mm.).
3. An equal volume (0.1 ml.) of each dilution of antigen is layered onto the serum.
4. Incubate at 37° for 2 hours and observe at 30 minute intervals for ring formation (precipitate at juncture of serum and antigen).
5. Shake tubes and incubate overnight at 4°C. The precipitate will settle out and can be read by gentle shaking of the tubes.
6. Controls of antigen with saline and serum with saline must be included and should show no precipitate.

COMPLEMENT FIXATION

The complement fixation test is based upon the observation that the combination formed between an antigen and its specific antibody has the property of uniting with complement. On the basis of this general law, complement can be used to detect the union of an antigen with its homologous or specific antibody. When a mixture of antigen and antibody is furnished with an exactly sufficient quantity of complement, all the complement is "fixed", or completely utilized in the reaction and none is left free in the fluid to take part in any other reaction between an antigen and its antibody which may be added subsequently for test purposes.

The test for such fixation is performed by placing together antigen, antibody and complement in suitable proportions, as determined by previous titrations, and subsequently testing for the disappearance of complement. If the complement is not fixed, it indicates that the antigen and antibody do not have the power to unite, or, in other words, that the antigen and antibody are not specifically related. On the other hand, the fixation of complement in the mixture indicates that the antigen and antibody have combined, because of their specific affinities.

In some cases the union of the complement with the antigen-antibody complex produces a solution or lysis of the antigen. In other

[1]See page VIII47-5–6.

cases no demonstrable lysis occurs, although the three substances, complement, antigen, and antibody, become united. If no obvious visible phenomenon accompanies the fixation of complement by a bacterial antigen and antibody, it becomes necessary to add to the primary mixture of complement, antigen, and antibody, an indicator capable of detecting whether the complement is fixed or is still free.

The only available indicator is an antigen-antibody mixture which undergoes visible change in the presence of free complement and shows no change in the presence of fixed complement. Such an indicator is a mixture of red blood corpuscles and a specific antibody for these. For convenience, sheep erythrocytes are most frequently used for this purpose. An antibody, called *hemolysin*, or anti-sheep-cell *amboceptor*, is prepared by immunizing an animal of a different species (usually rabbit) by means of injections of sheep's washed red corpuscles. This hemolytic amboceptor is a thermostabile antibody which retains its potency over long periods in suitable storage. When the amboceptor in the serum of the immunized animal (rabbit) reaches a suitable potency, the animal is bled, and the amboceptor-containing serum is preserved for subsequent use. Amboceptor is freed by heat from the complement in the serum of the immunized animal which produced it. The combination between red cells and specific amboceptor plus complement causes *hemolysis*, or laking of the cells.

A mixture of this antibody and the red corpuscles for which it is specific is used as an indicator of the degree of fixation of complement in any other antigen-antibody combination to which complement had been originally added in the right proportions. If the complement has been fixed by the formation of the first antigen-antibody combination, none will be left to bring about hemolysis of the red corpuscles: they will not be laked. But if complement is still free, the red blood cells will be hemolyzed when they are added with amboceptor to the original mixture. The first type of reaction, shown by absence of hemolysis, is called a positive reaction, indicating the specific union of the antigen and antibody being tested. The second type of reaction, hemolysis or laking of the red cells, is called a negative reaction, indicating that the original antigen-antibody mixture did not result in a specific combination.

Innumerable practical applications of the complement fixation test are made, especially in diagnosing various infectious diseases. Such applications do not fall within the scope of this MANUAL; but there are various ways in which essentially the same technic may be adapted for use in the pure culture study of bacteria. Antibodies, in general, are quite specific in their action; in other words, they will ordinarily unite only with the particular antigen inoculated into the animal in which the antibodies are produced, or else with some other related antigen. For this reason, the complement fixation test may be employed in pure culture study by producing antibodies to the various strains under investigation (by means of animal inoculation) and then determining the probable relationship of these strains by noting the action between the known antibodies and the sus-

pensions (antigens) of the various strains. If complement fixation occurs (indicating that such a union has taken place) it is assumed that the bacterium used as antigen in the test must have antigens in common with the bacterium used to produce the antibody.

The test requires *careful attention to detail* and the preparation of a number of accurately standardized serological reagents (antigen, antibodies or immune serum, complement, red corpuscles—usually those of a sheep—and antibodies to red corpuscles, known as hemolysin). A brief discussion of the methods of preparing these reagents is given below, as well as the methods of making the test. If greater detail is desired, it may be obtained by consulting standard text books on Immunology and Serology.

Materials Required: The glassware used for the complement fixation test, as well as for other serological reactions, should be chosen with care and kept scrupulously clean. Texts dealing with the Wassermann reaction describe suitable test tubes and pipettes A convenient tube is one measuring 100×10 mm. The pipettes should be serological pipettes: 10 ml. and 5 ml. pipettes graduated in 0.1 ml.; 1 ml. pipettes graduated in 0.1 ml.; and 0.2 ml. pipettes graduated in 0.01 ml. Suitable racks are necessary for holding the tubes.

PREPARATION OF REAGENTS FOR BACTERIAL COMPLEMENT FIXATION REACTION

(a). *Antigen.* With 0.85% NaCl solution ("saline") wash off the growth from a 24-hour agar slant culture of the organism to be used. The amount of saline necessary to make a satisfactory emulsion varies between 5 and 10 ml. depending upon the heaviness of growth. Shake well.[1] Filter through cotton. Heat in a water bath at 60°C. for 1 hour. Phenol, to make a 0.5% solution, may be added. This is not advisable, however, as it increases the anticomplementary action. This suspension may be kept for weeks in the cold without much loss of antigenic power.

For comparative work, the density of the emulsion should be standardized by nephelometric determinations or by a direct count of the number of organisms contained in 1 ml., as it is important to use approximately similar suspensions. All cell suspensions, including suspensions of bacteria, have the property of inhibiting the action of complement. This non-specific property is known as their "*anticomplementary action.*" The titration of the anticomplementary action of the antigen is given in a subsequent paragraph.

There are a number of other methods of preparing bacterial antigens some of which are better adapted to certain kinds of bacteria than the one given here. Extracts or solutions of bacteria and organisms obtained from broth or special culture media may be used. The Committee realizes the difficulties involved in preparing a satisfactory antigen, but feels that a complete treatise on this important subject is outside the scope of this MANUAL. The student must consult with instructors and refer to text books for more definite suggestions. A good antigen is the most difficult of all the required reagents to secure.

(b). *Immune Serum (Antibody).*[2] Immunize an animal against the organism to be studied by repeated injections of the organism. Rabbits are especially suitable for this purpose. Injections may be made into the marginal veins of the ears, intraperitoneally, or subcutaneously. For the injections, use light suspensions of the organism in 0.85% saline, made by washing off the culture from a 24-hour agar slant. As little as possible of the medium should be added to the same with the organism. Washed broth cultures can be employed in cases where it is desired to use an organism

[1] A preferable procedure would be the use of a shaking machine for two days; centrifuge to give a clear extract.

[2] See page VIII47-5-6.

which will not grow well on agar slants. Organisms requiring a carbohydrate for growth can be grown in sugar broth and then washed free of acid and used as antigen. Before the suspensions are injected, they should be heated for 1 hour at 60°C. On the first injection, use 0.5 ml. of this suspension. Increase the dose by increments of 0.5 ml. at intervals of 5 to 7 days. If the organism is not too virulent and the animal has not lost weight, the last few injections may be made with unheated suspensions of living organisms. About one week after the last injection, bleed the rabbit from the ear vein and obtain sufficient serum for a preliminary test to determine its potency. If this test shows that the serum contains antibodies in sufficiently high titer, bleed the rabbit from the heart, or in some manner which will provide as large an amount of serum as possible. After the collection of the serum, heat it at 56° C. for 1 hour to destroy complement, add 0.3% tricresol as a preservative, and store in sealed ampules or bottles.

It is not possible to lay down an invariable rule as to the total amount of antigen which should be injected to bring about a sufficient production of antibodies or to specify exactly the period of time required for the series of injections. Immune sera obtained after short periods of immunization are usually more specific than those obtained after long periods of immunization. By trial the amounts to be used in the final test can be determined; see p. VIII$_{47}$-16–17.

(c). *Complement.* Guinea pig serum furnishes an active and easily fixable complement. It is usually advisable to pool the sera from at least 3 guinea pigs weighing 1 to 2 pounds to obtain a sample of complement having average properties. Bleed the guinea pigs from the heart, removing 5 to 10 ml. of blood from each animal. Allow the blood to clot. Pipette off the serum and store in a sterile glass container in the refrigerator. The most potent complement can be obtained by allowing the clotted blood to stand overnight in a refrigerator before separating the serum. Complement rarely retains its potency longer than 3 days. It is essential to titrate it daily. Very fine work requires titration twice a day, keeping the complement in the refrigerator as much as possible when not actually being used. Complement preserved by the lyophile process or the cryochem process may be used: see Mudd *et al.* (1936), Ecker and Pillemer (1938).

(d). *Sheep's Red Blood Corpuscles.* With a veterinary needle, or a 19-gauge needle attached to a 50 ml. syringe, withdraw 10 to 50 ml. of blood from the external jugular vein of a sheep. Place the blood at once in a sterile flask containing glass beads. Shake for 15 minutes to defibrinate, and filter through gauze or absorbent cotton to remove the fibrin. Instead of defibrinating in this manner, the blood may be mixed with an equal volume of 0.85% saline containing 2% sodium citrate. This prevents coagulation and makes it unnecessary to remove the fibrin. Wash the cells 3 times in 0.85% saline. This is done by centrifuging the cells at about 1500 r. p. m. for 10 to 15 minutes. Pipette off the supernatant fluid and add as much fresh saline as the amount removed. Mix well and repeat the process twice. Final centrifugation should be at 1800 r. p. m. in order to pack the cells. After the final washing, carefully remove the supernatant saline without disturbing the packed sediment of cells. With this sediment make a 2.5% suspension of the red cells in saline by adding 2.5 ml. of the packed cells to 97.5 ml. of saline. If it is desirable to keep the cells longer than 3 days, 0.1 ml. of a 1–10 dilution of 40% formaldehyde may be added to 8 ml. of blood. This mixture as well as any other suspension of blood cells should be kept in the refrigerator until used. Before use, the cells should be washed 3 times in saline (or until supernatant fluid is clear and colorless). For accurate work it is best to use fresh cells.

For hemolysin production, red cells which have not been treated with formalin should be used.

(e). *Amboceptor (Anti-sheep-red-cell Hemolysin).*[1] Very strong hemolysin may be obtained by the following method: Two healthy rabbits are given intravenous injections of undiluted and unpreserved washed sheep's corpuscles according to the following schedule: 1st day, 0.5 ml. packed erythrocytes; 3rd day, 1.0 ml.; 5th day, 1.0 ml.; 7th day, 1.0 ml.; 11th day, 1.5 ml.

Eight days after the last injection a trial bleeding is made from the marginal ear vein. If the serum is found sufficiently potent the rabbits are bled to death or enough blood is taken from the ear vein as is desired for stock hemolysin. The latter method should yield all the serum needed, at least if the bleeding is repeated on two or three successive days, and if both ears are used.

[1]See also Beattie, (1934); von Daránvi, J., (1928); Stafseth (1932); Ulrich and McArthur (1942); Sawyer and Bourke (1946).

The serum is allowed to separate from the clot, pipetted off, and treated with 0.4% phenol, 0.3% tricresol, or an equal amount of 50% neutral glycerol. The potency will be retained for many months, when stored in the refrigerator. Titrations should be made at intervals, however, not exceeding three or four months.

The titration of hemolytic amboceptor, using a constant amount of complement, is discussed below. The hemolytic titer (unit) should be at least 0.25 ml. of a 1–1000 dilution.

If the amboceptor does not have such potency as this, it is advisable to continue the injection of increasing amounts of the sheep cells. For sharp reactions, in which a minimal amount of complement can be used, and to have an amboceptor which can be diluted well beyond its agglutinative effect upon red corpuscles, it is advisable to prepare an amboceptor with a high titer.

TITRATION OF REAGENTS

Before proceeding with the test, the relative strength of each of the reagents must be known and the amounts necessary for a successful test determined. This process is known as *titration*. All the reagents, with the exception of the red corpuscles, and the specific immune serum (antibody), should be titrated before any test is conducted. Whenever a freshly prepared reagent is used, it must be titrated. Daily titrations of complement must be made when tests are done each day.

Titration of Amboceptor (Hemolysin). In this titration, decreasing amounts of amboceptor are mixed with a constant amount of complement and added to sheep's red corpuscles to determine the smallest amount of amboceptor which will cause hemolysis of the sheep cells. (To prepare a specimen of complement having good average properties, mix the blood serum obtained from bleeding at least 3 normal guinea pigs.) Dilute this complement 1 to 10 with saline. It is advisable to keep the flask containing complement on ice or in ice water, to prevent the deterioration which takes place appreciably, even at room temperature. Next make up the following series of dilutions of the anti-sheep amboceptor: 1–100, 1–200, 1–400, 1–1600, 1–3200, 1–6400. Prepare a 2.5% suspension of washed red corpuscles (sheep) as described above. Set up the tubes for this titration according to the following protocol. (Table 3)

TABLE 3

TITRATION OF HEMOLYTIC AMBOCEPTOR—WITH RESULTS IN A TYPICAL INSTANCE

Tube	Amboceptor		Complement 1–10	Sheep Cells 2.5% susp.	Incubate in water bath at 37°C. for 15 mins., with shaking	Hemolysis
	Dilution	Amount				
1	1–100	0.25 ml.	0.25 ml.	0.25 ml.		Complete
2	1–200	0.25 ml.	0.25 ml.	0.25 ml.		Complete
3	1–400	0.25 ml.	0.25 ml.	0.25 ml.		Complete
4	1–800	0.25 ml.	0.25 ml.	0.25 ml.		Complete
5	1–1600	0.25 ml.	0.25 ml.	0.25 ml.		Complete
6	1–3200	0.25 ml.	0.25 ml.	0.25 ml.		Partial
7	1–100	0.25 ml.	.....	0.25 ml.		None
8	.....	.....	0.5 ml.	0.25 ml.		None

Tubes 7 and 8 are controls used to show whether or not either the amboceptor or complement is hemolytic. If either is hemolytic, that reagent should be discarded. Some specimens of complement are quite hemolytic.

After the mixtures are made, place the rack containing the tubes in the water bath at 37° C. and incubate them for 15 min., shaking repeatedly. At the end of the period of incubation, note hemolysis. The tube containing the highest dilution of the amboceptor which produces complete hemolysis of the cells (tube 5 in instance illustrated in Table 3) denotes the titer of the amboceptor. In this system, 0.25 ml. of that dilution of the amboceptor is called *one unit* of the amboceptor. *This unit now becomes a fixed standard, as the amboceptor is a stable substance.* In subsequent titrations of comple-

ment and in the final test, use 3 units of amboceptor (hemolysin). *Example:* If, in the above titration, 0.25 ml. of a 1–1600 dilution of the amboceptor produced complete hemolysis of 0.25 ml. of the 2.5% suspension of sheep cells, 3 units of amboceptor would be contained in 0.25 ml. of a 1–533 dilution of the stock amboceptor hemolysin serum, or a 1–265 dilution of amboceptor serum which has been put up with an equal part of glycerin.

Titration of Complement. Since the activity of complement in the serum of different guinea pigs varies, and as the activity of any sample of complement changes on standing, this reagent must be titrated at least once daily. The activity of the sample to be used is, therefore, titrated in terms of the arbitrarily established unit of hemolytic amboceptor. In general, there is a reciprocal relationship between complement and amboceptor. Within certain limits, hemolysis of a given amount of red corpuscles can be produced by mixtures containing more of complement and less of amboceptor, and vice versa. The purpose of the following titration is to determine by dilution the smallest amount of complement which will cause complete hemolysis of 0.25 ml. of 2.5% sheep red cells in the presence of 3 units of amboceptor. After having obtained and mixed the serum from at least 3 guinea pigs, dilute the complement 1–10 and proceed as in Table 4.

TABLE 4

TITRATION OF COMPLEMENT—WITH RESULTS IN A TYPICAL INSTANCE

Tube	Complement; guinea pig serum diluted 1–10	Amboceptor 3 units	Sheep Cells 2.5% susp.	Saline	Incubate 15 mins., shaking repeatedly at 37°C. in water bath	Hemolysis
1	0.15 ml.	0.25 ml.	0.25 ml.	0.60 ml.		Complete
2	0.14 ml.	0.25 ml.	0.25 ml.	0.61 ml.		Complete
3	0.13 ml.	0.25 ml.	0.25 ml.	0.62 ml.		Complete
4	0.12 ml.	0.25 ml.	0.25 ml.	0.63 ml.		Complete
5	0.11 ml.	0.25 ml.	0.25 ml.	0.64 ml.		Complete
6	0.10 ml.	0.25 ml.	0.25 ml.	0.65 ml.		Complete
7	0.09 ml.	0.25 ml.	0.25 ml.	0.66 ml.		Partial
8	0.08 ml.	0.25 ml.	0.25 ml.	0.67 ml.		None
9	0.25 ml.	.....	0.25 ml.	0.75 ml.		None
10	.....	0.25 ml.	0.25 ml.	0.75 ml.		None
11	.....	.....	0.25 ml.	1.00 ml.		None

Saline is added to the tubes in this series to bring the volume of fluid in each tube up to 1.25 ml., the amount of fluid used in the final test. Tube 9 is the control for the hemolytic activity of the complement alone; tube 10 serves a similar purpose as an amboceptor control, and tube 11 is a control for the isotonicity of the saline solution.

At the end of the 15 min. period of incubation, note the last tube showing complete hemolysis. This gives the smallest amount of the 1–10 dilution of complement which will cause the hemolysis of 0.25 ml. of a 2.5% suspension of sheep cells in the presence of 3 units of amboceptor. In the final test, use 1.5 times as much complement as in this tube. *Example:* If, as illustrated in the table, the smallest amount of complement causing hemolysis were 0.1 ml. of the 1–10 dilution, use 0.15 ml. of a 1–10 dilution of complement in the final test. With different specimens of complement, it may be necessary to use a different series of amounts to arrive at the exact titer of the complement. This method of titration is devised to permit the use of minimal amounts of complement in the final test.

Titration of the Antigen. After the bacterial antigen has been prepared by emulsifying the culture in saline, it is necessary to find out by titration three of its properties. These are: (a) the ability of the antigen alone to inhibit the action of complement, called the anticomplementary action of the antigen, (b) the hemolytic properties of the antigen, and (c) the capacity of the antigen to fix complement in the presence of its specific antiserum, called the binding power of the antigen. These properties can be determined by the procedure outlined in Table 5.

Interpretation of Results. It will probably be found that most bacterial suspensions are anticomplementary, and some are slightly hemolytic. In the first series of tubes

in this protocol note the first tube in which complete hemolysis occurs. This denotes the end of the anticomplementary action of the antigen. In the final test do not use more than one-third of the amount of the antigen which was found to be anticomplementary. *Example:* If 0.5 ml. of the antigen were found to be anticomplementary, do not use more than 0.17 ml. of this bacterial suspension in the final test. *It is to be em-*

TABLE 5

TITRATION OF ANTIGEN—WITH RESULTS IN A TYPICAL INSTANCE

Property investigated	Tube	Antigen	Antiserum dilution 1–25	Complement dilution 1–10	Saline		Add after 1st incubation: Amboceptor 3 units	Add after 1st incubation: Sheep Cells 2.5% susp.		Hemolysis
		ml.	ml.	ml.	ml.		ml.	ml.		
Anti complementary action	1	0.5	...	0.15	0.1	Incubate 1 hour at 37°C. in water bath	0.25	0.25	Incubate 15 mins. at 37°C. in water bath	Partial
	2	0.4	...	0.15	0.2		0.25	0.25		Complete
	3	0.3	...	0.15	0.3		0.25	0.25		Complete
	4	0.2	...	0.15	0.4		0.25	0.25		Complete
	5	0.1	...	0.15	0.5		0.25	0.25		Complete
	6	0.05	...	0.15	0.55		0.25	0.25		Complete
Hemolytic action	7	0.5	...	...	0.5		...	0.25		None
	8	0.1	...	...	0.9		...	0.25		None
Binding power	9	0.25	0.25	0.15	0.1		0.25	0.25		None
	10	0.1	0.25	0.15	0.25		0.25	0.25		None
	11	0.075	0.25	0.15	0.275		0.25	0.25		None
	12	0.05	0.25	0.15	0.3		0.25	0.25		None
	13	0.025	0.25	0.15	0.325		0.25	0.25		Partial
	14	0.01	0.25	0.15	0.34		0.25	0.25		Complete
Controls	15	...	0.25	0.15	0.35		0.25	0.25		Complete
	16	...	0.25	...	0.75		...	0.25		None

phasized again that the amounts set down in Table 5 are arbitrarily chosen. A different series of amounts might be found more suitable for different reagents. There should be no hemolysis in tubes 7 and 8 showing that the antigen alone does not lake the red cells. In the third series, tubes 9 to 14, absence of hemolysis denotes fixation or binding of the complement. The last tube in this series showing complete absence of hemolysis indicates the smallest amount of the antigen which will fix complement in the presence of the constant amount of its antiserum used in this titration. The fixing power of the antigen should be at least 10 times as great as its anticomplementary action.

The titration of the antigen should be made whenever a new bacterial suspension is prepared, or at intervals of 3 to 4 weeks if old suspensions are kept on hand.

PROCEDURE FOR COMPLEMENT FIXATION TEST

The amounts of reagents used in the final test for complement fixation are those which have been found to be appropriate from the preliminary titrations described above. Stated in the form of a general protocol, the test should be set up as follows:

Tube 1. Amount 1 of immune serum plus complement plus antigen.

Tube 2. Amount 2 of immune serum plus complement plus antigen.

Tube 3. Amount 3 of immune serum plus complement plus antigen.

These tubes constitute the test for complement fixation. A careful series of controls is necessary, as follows:

Tube 4. (Anticomplementary serum control): Double the largest amount of antiserum plus complement.

Tube 5. (Anticomplementary antigen control): Twice the amount of antigen used in test plus complement.

Tube 6. (Hemolytic system control): Complement alone.

Tube 7. (Saline control): Saline alone.

Add sufficient saline so that the total volume of fluid, when all ingredients are in the tubes, will be 1.25 ml.

TABLE 6

COMPLETE COMPLEMENT FIXATION TEST—WITH RESULTS IN A TYPICAL INSTANCE

Tube	Antigen	Immune Serum	Complement 1–10 dil.	Saline	Incubation at 37°C. for 1 hour in water bath.	ADD AFTER 1ST INCUBATION		Incubate at 37°C. for 15–30 minutes in water bath.	Hemolysis
						Amboceptor 3 units	Sheep Cells 2.5% susp.		
1	0.25 ml.	0.1 ml.	0.15 ml.	0.25 ml.		0.25 ml.	0.25 ml.		None
2	0.25 ml.	0.05 ml.	0.15 ml.	0.3 ml.		0.25 ml.	0.25 ml.		None
3	0.25 ml.	0.01 ml.	0.15 ml.	0.34 ml.		0.25 ml.	0.25 ml.		Partial
4	.....	0.2 ml.	0.15 ml.	0.4 ml.		0.25 ml.	0.25 ml.		Complete
5	0.5 ml.	.....	0.15 ml.	0.1 ml.		0.25 ml.	0.25 ml.		Complete
6	.....	.....	0.15 ml.	0.6 ml.		0.25 ml.	0.25 ml.		Complete
7	.....	.....	.....	1.0 ml.		.....	0.25 ml.		None

The optimum temperature of incubation of the mixtures for complement fixation varies under different conditions. The test is in some cases more sensitive when these mixtures are kept in the refrigerator at 5–10°C. for 4 hours. For most purposes incubation in a water bath at 37°C. for 1 hour, as given in the protocols, is satisfactory.

After this incubation, add to all tubes except tube 7, 3 units of amboceptor contained in 0.25 ml. of the diluted amboceptor serum and to all tubes add 0.25 ml. of 2.5% sheep cells. Shake well, and incubate them again, for 15–30 minutes, depending upon the rate of hemolysis in the control tubes.

At the end of the second period of incubation, note the results. The partial or complete absence of hemolysis in any of the first 3 tubes denotes fixation of complement, indicating union between the antigen and antiserum. Hemolysis in these tubes indicates lack of fixation or a negative reaction.

Tubes 4, 5 and 6 should show complete hemolysis, indicating that the serum and antigen are not anticomplementary and that the hemolytic system is working properly.

There should be no hemolysis in tube 7, showing that the salt solution is isotonic with the sheep cells. When several tests are made at the same time with the same immune serum, the control tubes 4, 6 and 7 need not be repeated. It is necessary, however, to add an anticomplementary antigen control whenever a different antigen is used, and another anticomplementary serum control whenever a different serum is used.

Example: A specimen protocol, giving amounts of the reagents presumed to have been decided upon after the preliminary titrations described above, is given in Table 6. (N. B. *The amounts stated here are arbitrary amounts and are not to be applied to an actual test unless justified by previous titrations.*)

A measurement of the titer of an immune serum can be made by this test. The specificity of the serum can be judged only by testing it in this manner against other antigens. In interpreting the results of this test for the purpose of pure culture studies, it may be assumed that when an organism causes complement fixation in any of the tubes 1 to 3 with an antiserum produced by the immunization of an animal against another organism, the two organisms have common antigens. The results of complement fixation tests, however, must not be regarded as a basis for exact determinations of identity, as certain antigens may show positive reactions with the sera of entire groups. The test is often more indicative of group relationships than of identities.

TITRATION OF TOXINS, TOXOIDS AND ANTITOXINS

(Flocculation method)

An unknown toxin or toxoid may be titrated with an antitoxin of known value or an unknown antitoxin with a toxin or toxoid of known value *in vitro*. In serial mixtures of the two, there first occurs a cloudiness followed by a precipitate in some of the tubes, and finally a definite flocculation in one tube which is taken as the tube containing the "indicating mixture". The flocculation in this first tube may be followed by flocculation in other tubes about it within a short time. *The "indicating mixture," however, is always the initial tube to flocculate and must be watched for rather cautiously.* From this "indicating mixture" is calculated the *flocculating unit* of the toxin, which has been designated *Lf*. *The Lf may be defined as the amount of toxin equivalent to 1 unit of antitoxin as established by flocculation.* There is no complete agreement or relationship between the M.L.D., Lo, L, and the Lf values of a toxin. The first three of these units have been designated as "*in vivo* units" and the fourth as an "*in vitro* unit". Flocculation may occur at any temperature up to 55°C., above which the reaction becomes irregular and often completely inhibited. A temperature of 40° to 50°C. is the most suitable zone. The time of incubation and of flocculation vary with different toxins

and different antitoxins. The tubes must be observed every 15 minutes at these temperatures in order to observe the tube in which initial flocculation occurs. The reaction is probably due to a combination of the antigenic portion of the toxin and the antitoxin. The "floc" formed is composed of both toxin and antitoxin in dissociable union—dissociated by heat or sodium iodide.

There is no difficulty in obtaining flocculation with the first fraction in the concentration of antitoxins but later fractions may not flocculate. Concentrated toxoids likewise may not flocculate. The method of titration has been applied mainly to diphtheria and tetanus toxins, toxoids, and antitoxins. It has been applied to others and may be applied still further.

PROCEDURE

To a series of ten test tubes (4″×½″) add serially amounts of antitoxin, differing by 0.005 ml. (or 0.001 ml.) from tube to tube. This may be done by means of a 0.2 ml. pipette graduated in 0.01 ml.(or 0.001 ml.) or, if greater accuracy is desired, by means of a Trevan micro-syringe. Add to each tube from a 10 ml. pipette 2 ml. of the toxin or toxoid to be titrated for its Lf value. The tubes are now shaken, placed in a *water bath* at 40 to 50° C. and observed every 15 minutes for the first appearance of flocculation.

TABLE 7

TITRATION OF A DIPHTHERIA TOXIN WITH RESULTS IN A TYPICAL INSTANCE

(Floeculation method)

Tube No.	Antotoxin No. 1347	Toxin No. 16304	Time in minutes				Indicating Mixture
			15	30	45	60	
1	0.020 ml.	2.0 ml.	..	..	..	..	
2	0.025 ml.	2.0 ml.	..	..	..	..	
3	0.030 ml.	2.0 ml.	..	P	P	P	
4	0.035 ml.	2.0 ml.	..	P	P	P	
5	0.040 ml.	2.0 ml.	C	P	P	P	
6	0.045 ml.	2.0 ml.	C	P	P	P	
7	0.050 ml.	2.0 ml.	C	P	P	F	
8	0.055 ml.	2.0 ml.	C	P	F	F	8th tube in 45 minutes.
9	0.060 ml.	2.0 ml.	C	P	P	F	
10	0.065 ml.	2.0 ml.	C	P	P	P	

C = Cloudiness; P = Precipitate; F = Flocculation.
Temperature of the water bath 50°C.
Depth of the tubes in water—water ¾ distance to top of the liquid in the tubes.
Size of tubes 4″×½″ (inside dimensions).
Antitoxin used in above titration contained 425 units per ml. (or 1 unit is contained in 0.00235.)

In Table 7 is given a protocol for the titration of an unknown diphtheria toxin with the results obtained. For greater accuracy the toxin would be retitrated using 0.050 to 0.60 ml. of antitoxin with differences of 0.001 ml. between tubes.

Calculation of the typical instance given in Table 7 is as follows:

2 ml. of toxin flocculated with 0.055 ml. of antitoxin ("indicating mixture")
0.00235 of the antitoxin contains 1 unit
Since the Lf = the amount of toxin that will flocculate with 1 unit of antitoxin
Therefore, $2 : 0.055 = x : 0.00235$

$x = 0.086$ which is the Lf of toxin No. 16304 (or 11.6 flocculating units per cc.)

For details on the titration of toxins and antitoxins in animals the reader is especially referred to the recent publication of Gershenfeld (1939) and to Wadsworth's book (1947).

REFERENCES

Beattie, Margaret. 1934. A new method for the production of antisheep hemolysis. J. Lab. and Clin. Med., **19**, 666–667.

Ecker, Enrique E. and Pillemer, L. 1938. An inexpensive method for the dehydration and preservation of complement and other biological material. Am. J. Pub. Health, **28**, 1231–1232.

Gershenfeld, Louis. 1939. Biological Products. Romaine Pierson Publishers, Inc., New York.

Kolmer, John A. and Boerner, Fred. 1938. Approved Laboratory Technic. Fourth edition. D. Appleton-Century Co., New York.

Landsteiner, K. 1946. The Specificity of Serological Reactions. Harvard University Press, Cambridge, Massachusetts.

McFarland, John. 1907. The Nephelometer: An instrument for estimating the numbers of bacteria in suspensions used for calculating the opsonic index and for vaccines. J. Am. Med. Assoc., **49**, 1176–1178.

Marrack, J. R. 1938. Chemistry of Antigens and Antibodies. Medical Research Council, London.

Mudd, Stuart; Flosdorf, Earl W.; Eagle, Harry; Stokes, Joseph; and McGuinness, Aims C. 1936. The preservation and concentration of human serums for clinical use. J. Am. Med. Assn., **107**, 956–959.

Ramon, G. 1922. Flocculation dans un melange neutre de toxine antitoxine diphtherique. Compt. Rend. Soc. Biol., **86**, 661–663

Sawyer, H. P. and Bourke, A. R. 1946. Antisheep Amboceptor Production with Elimination of Rabbit Shock. J. Lab. and Clin. Med., **31**, 714–716.

Stafseth, H. J. 1932. On the preparation of hemolytic and precipitating sera. Science **76**, 444.

Trevan, J. S. 1922. An apparatus for the measurement of small quantities of fluid. Lancet I, 786.

Ulrich, Catherine A. and McArthur, Francis X. 1942. An improved method for the production of antisheep hemolysin. Am. J. Clin. Path. (Clin. Sect.) **6**, 84-85.

von Daranyi, J. 1928. Methods of obtaining and preserving antibodies. J. Immunol., **15**, 521–526.

Wadsworth, Augustus B. 1947. Standard Methods. Williams and Wilkins Co., Baltimore.

Zinsser, Hans; Enders, John F.; and Fothergill, Le Roy D. 1939. Immunity. Principles and Application in Medicine and Public Health. The Macmillan Company, New York.

LEAFLET IX

OF

MANUAL OF METHODS FOR
PURE CULTURE STUDY OF BACTERIA

THE MEASUREMENT OF pH, TITRATABLE ACIDITY, AND OXIDATION-REDUCTION POTENTIALS

10th EDITION
Pure Culture Study of Bacteria, Vol. 16, No. 3–4
SEPTEMBER, 1948

Completely Revised by Barnett Cohen

LEAFLET IX

THE MEASUREMENT OF pH, TITRATABLE ACIDITY, AND OXIDATION-REDUCTION POTENTIALS[1]

The Measurement of pH

Originally, pH was defined as the logarithm of the reciprocal of the hydrogen ion concentration. However, certain assumptions regarding indeterminate factors enter the theoretical treatment of any method of measuring this quantity. It is now recognized that the pH scale is standardized on a basis that is arbitrary with respect to a small and indeterminate uncertainty, although any pH number closely approximates the logarithm of the reciprocal of the corresponding hydrogen ion *activity*. The activity of any substance is virtually the product of that substance's molar concentration and a factor, called the activity coefficient. This factor expresses the departure from that behavior which would obtain were there no van der Waals and Coulomb (attraction and repulsion) forces operating.

The common methods for the measurement of pH are of two types: (1) potentiometric, and (2) colorimetric. The theoretical and practical aspects of the subject are treated extensively in the monograph by Clark (1928).

POTENTIOMETRIC METHODS

The several potentiometric methods to be cited depend upon the fact that the pH of a solution suitably incorporated in a so-called half-cell is proportional to the electric potential difference established between this half-cell and some reference half-cell used as a standard.

The Hydrogen electrode method. This is regarded as the basic experimental method whereby the various other methods are standardized. It consists in the measurement of the potential difference (emf) established under conditions of maximum work between the "hydrogen half-cell", or "hydrogen electrode", and a calomel or other half-cell which is employed as a working standard.

The hydrogen half-cell consists of a suitable vessel provided with (*a*) a platinum foil electrode, coated with platinum-black, which is immersed or intermittently dipped in the solution to be measured, and (*b*) an inlet and outlet for oxygen-free hydrogen to saturate both solution and electrode at atmospheric pressure.

A convenient reference half-cell is the "saturated calomel electrode" which consists of a vessel containing a layer of purified mercury covered with a paste of calomel (Hg_2Cl_2), mercury, and saturated KCl solution; the calomel paste is layered with crystals of KCl, and the rest of the vessel is filled with saturated KCl solution which has been saturated with calomel. A platinum wire provides the electrical lead to the mercury of the calomel cell, and a siphon containing saturated KCl solution provides liquid junction with the solution to be measured in the hydrogen half-cell.

In the normal hydrogen half-cell, which provides the standard of potential for all measurements of potential in electrochemistry, the hydrogen partial pressure is one

[1]This presentation is confined to the brief description of general procedures that may be applied in the bacteriological laboratory. For theoretical discussions and the elaboration of detail, the reader should consult the texts, monographs, and original references cited.

normal atmosphere and the hydrogen ions are at unit activity. The potential difference between electrode and solution in the normal hydrogen half-cell is assumed to be zero at all temperatures.

In standardizing the pH scale by means of measurements with a cell composed of a hydrogen half-cell and a saturated KCl calomel half-cell, it is customary to ignore the small and indeterminate liquid junction potential between the saturated solution of KCl and the solution in the hydrogen half-cell.

The combination of the two half-cells to make an electric cell is indicated schematically as follows:

$(Pt)H_2$; H^+ in solution X	Sat. KCl	Sat. KCl; Hg_2Cl_2; Hg (Pt)
Hydrogen electrode	KCl bridge	Sat. calomel (reference) electrode

For a pH determination, purified hydrogen is bubbled through the test solution to saturate it and the platinized platinum electrode until equilibrium is attained as indicated by constancy of the emf determined potentiometrically between the metal terminals of the hydrogen and the calomel half-cells. The observed emf, in volts[2], is converted to pH by the following equation, where T is the absolute temperature.

$$pH = \frac{\text{Observed emf} - \text{Emf of calomel cell}}{0.000,198,322\ T} = \frac{E_h}{0.000,198,322\ T} \qquad (1)$$

For this equation to be applicable, the temperature must be constant. For precise measurements, a correction must be made for any departure of the hydrogen partial pressure from one atmosphere. The correction seldom exceeds 0.001 volt (0.017 unit of pH) for the ordinary ranges of barometric pressure and vapor pressures of solutions.

As indicated by equation 2,

$$\frac{-\triangle E_h}{\triangle pH} = 0.000,198,322\ T \qquad (2)$$

the slope of the straight line relating potential to pH is a constant dependent on the absolute temperature. For example, at 25°, the potential of the hydrogen electrode becomes more negative by 0.0591 volt[2] for each unit increase in pH. Values of this constant at certain temperatures are shown as constant "A" on p. IX48-4.

Standardization of the saturated calomel half-cell. For ordinary measurements, the values at different temperatures of the saturated calomel half-cell, referred to the normal hydrogen half-cell, are as follows:

°C	E_{cal}	°C	E_{cal}
20	0.250 v.	35	0.238 v.
25	0.246	38	0.236
30	0.242	40	0 234

The potential of this half-cell after continued use may change as a result of dilution

[2]The electrical units employed in this leaflet are based on the "international" system in which, according to the National Bureau of Standards, 1 international volt (U. S.) equals 1.00033 absolute volts. The Bureau has announced that, as of January 1, 1948, absolute electrical units will supersede international units.

However, the effect of this new convention for potentiometry is to introduce changes which may be regarded as negligibly small in ordinary measurements of pH and oxidation-reduction potentials. For example, in equation 2, $-\triangle E_h/\triangle pH$ equals 0.05912 international volt and 0.05914 absolute volt, at 25°C (298.1° absolute).

and contamination, and it is advisable to check its value regularly as a routine procedure.

The precise standardization of the calomel half-cell is discussed in detail by Clark (1928). It consists in measuring the potential of this half-cell against the hydrogen electrode in a solution of known hydrogen ion activity or against other carefully constructed half-cells of reproducible, known potential. For measurements of ordinary precision, the quinhydrone electrode (see below) in 0.1 N HCl can serve for standardization of the calomel half-cell.

The quinhydrone electrode. Ignoring refinements and minor details, we may state that the potential of a noble metal electrode in an acid or neutral solution saturated with quinhydrone varies linearly with the pH of the solution; and this so-called quinhydrone electrode may, therefore, be used to measure the pH of such solutions.

The linear relationship of potential to pH holds *only for acid and neutral solutions* to about pH 8. In more alkaline solutions two effects disturb this regularity. One is the ionization of the reductant, and the other is deterioration of the components of the system.

The quinhydrone electrode within its range of usefulness, may often be employed in cases where the hydrogen electrode cannot be applied. It comes to equilibrium rapidly, and its manipulation is simple and convenient. Consult Clark (1928) for fuller details.

Its utilization may be illustrated in the standardization of the saturated calomel half-cell. The potential, E_{cal}, of this half-cell is to be determined relative to that of a standard solution of fixed pH and saturated with quinhydrone, e.g., 0.1 M HCl, the pH of which is 1.082 at 38°. This is done with purified quinhydrone and accurately prepared HCl solution as follows. Place about 5 ml. of the standard HCl solution in a suitable electrode vessel. Add 50 to 100 mg. of quinhydrone crystals to saturate the solution; some quinhydrone in the solid phase must be present. Insert a clean platinum or gold electrode preferably in contact with the solid phase at the bottom of the vessel. Then join this half-cell with the calomel half-cell by means of a siphon containing saturated KCl solution, bring the system to constant temperature, and measure the potential which should reach a constant value in a few minutes.

The observed potential, E_{obs}, is related to the potential of the calomel cell, E_{cal}, as follows:

$$E_{cal} = E_q - E_{obs} - A.pH \qquad (3)$$

E_q and A are constants at any given temperature, and have the following values:

°C	E_q	A
20	0.7029	0.0581
25	0.6992	0.0591
30	0.6955	0.0601
35	0.6918	0.0611
38	0.6896	0.0617

For example, at 38°, with a quinhydrone electrode in 0.1 M HCl,

$$E_{cal} = 0.6896 - E_{obs} - (0.0617 \times 1.082) \qquad (4)$$

from which the value of $E_{cal.}$ can be calculated after substitution of the experimentally determined value of E_{obs}.

To determine the pH of an unknown solution, proceed as above except that the unknown solution is substituted for the standard HCl.

The "glass electrode". Under suitable conditions, a properly prepared thin membrane of special glass separating two solutions of different pH exhibits an electric potential that is proportional to the difference in pH of the solutions. Based on this property, a device called the glass electrode is now widely used for the comparative determination of pH.

The glass probably most generally employed is that known as Corning No. 015; Beckman type E glass has been advocated for alkaline solutions (pH 9 to 14) because of its low sodium error as compared with that of glass 015.

One of the common forms of the glass electrode consists of a tube of the glass terminating in a thin-walled bulb which contains an electrode of definite potential in a solution of fixed pH. A combination of electrode and buffer solution frequently employed is a platinum wire, silver-plated and then coated with AgCl, in a half-cell containing 0.1 M HCl. For the construction, operation, and theory of the glass electrode, consult Dole (1941).

The carefully rinsed bulb of the electrode, after seasoning in water or buffer solution, is immersed in the solution to be tested and coupled through a saturated KCl liquid junction with the saturated calomel half-cell as indicated schematically below,

Ag; AgCl; HCl (0.1 M) | Glass membrane | Solution X | KCl (sat.); Hg_2Cl_2; Hg

all parts of the cell being maintained at a uniform temperature. The potential difference between the terminals of this cell can be related to the pH of solution X if the glass electrode has been standardized in buffer solutions of known pH.

Standardization of the glass electrode. The potential of a properly functioning glass electrode should vary linearly with pH, from about pH 1 to 9, in solutions of low salt content (up to 0.1 M). For this range, therefore, the electrode requires standardization in buffer solution at one point of pH, but preferably at two, within this linear range. Standard buffer solutions convenient for this purpose may be selected from Tables 1 and 3.

TABLE 1

SOME STANDARD BUFFER SOLUTIONS

Solution	pH	
	25°	38°
0.1 M HCl	1.085	1.082
0.01 M HCl, 0.09 M KCl	2.075	2.075
0.05 M Acid potassium phthalate	4.000	4.015
0.025 M KH_2PO_4, 0.025 M $Na_2HPO_4 \cdot 2H_2O$	6.855	6.835
0.05 M $Na_2B_4O_7 \cdot 10H_2O$	9.180	9.070

Such standardization should be performed at least daily; preferably, it should be done immediately before a measurement. As occasion requires, a series of buffer solutions of known pH should be used to establish more carefully the linearity of response of the electrode.

In solutions more alkaline than about pH 9, the 015 glass electrode responds also to cations other than H ions, the potential being influenced by the activity and kind of such cations. Sodium and lithium ions produce the most marked effects, potassium and bivalent cations smaller effects. When working under these conditions, it is advisable to standardize the electrode with known buffer solutions of about the same composition and of pH closely above and below the pH of the sample being tested.

The standardization for linearity of response from pH 1 to 9 is a necessary check on the operation of the glass electrode, since its results are comparative, not absolute. The slope, $-\triangle E_h/\triangle pH$, should be not merely constant at any temperature but also equal or closely equal to 0.000,198,322 T (the values for this constant are shown under A on p. 4). Obviously, a "pH-meter" with its pH scale adjusted to the theoretical slope for a given temperature cannot give correct readings at all points from pH 1 to 9 if its glass electrode follows a significantly different slope at the same temperature. For a brief discussion of the effects of temperature, see Clark (1948).

Cleaning of the glass surface, by immersion in a hot mixture of concentrated nitric and sulfuric acids followed by soaking in water, may restore a sluggish or erratic electrode to normal functioning. A somewhat drastic procedure that may be effective is to dip the glass electrode for a second or two in dilute HF or in a 20% solution of ammonium bifluoride and then to wash it thoroughly in water. If the electrode still behaves erratically, it should be discarded. For such an emergency, it is highly advisable to have available a reserve electrode. This may obviate any mistaken tendency to carry on with an electrode of doubtful reliability.

The instructions accompanying the various glass-electrode "pH-meters" now on the market are usually sufficient to aid the user in tracing out sources of trouble and error in operation. A major source of trouble is electrical leakage due to accumulation of films of moisture at critical parts of the circuit; and perhaps the most frequent sites of such accumulation are the electrode support and lead, both of which are apt to be spattered with water or salt solution during careless manipulation.

The glass electrodes now available are fairly rugged and easily adaptable to use under a variety of conditions and on different types of biological material (*e.g.*, liquid and "solid" culture media). Measurements with an accuracy of 0.05 pH may be made rapidly in poorly buffered, colored, or turbid solutions, and in blood or serum. The monograph by Dole (1941) discusses many of its uses.

THE COLORIMETRIC METHOD

The colorimetric method of measuring pH makes use of acid-base indicators, which, within certain limits, vary in color with the pH of the solution. Such indicators are compounds capable of existing in solution as conjugate proton (H-ion) donor and proton acceptor, with one of the conjugate pair differing in color from the other. The relation of these two forms to pH is defined by the equation

$$pH = pK' + \log \frac{[\text{proton acceptor}]}{[\text{proton donor}]} \qquad (5)$$

in which brackets represent concentrations, and pK′ (= − log K′) is called the apparent ionization exponent of the indicator's proton donor-acceptor system. Simple calculations, using, for example, 0.8, 0.5 and 0.3 as values for the ratio [proton acceptor]/[proton donor] at each of the pK′ values 3, 6, and 9, will show that indicators with different pK′ values cover different ranges of pH. (See Fig. 1). For

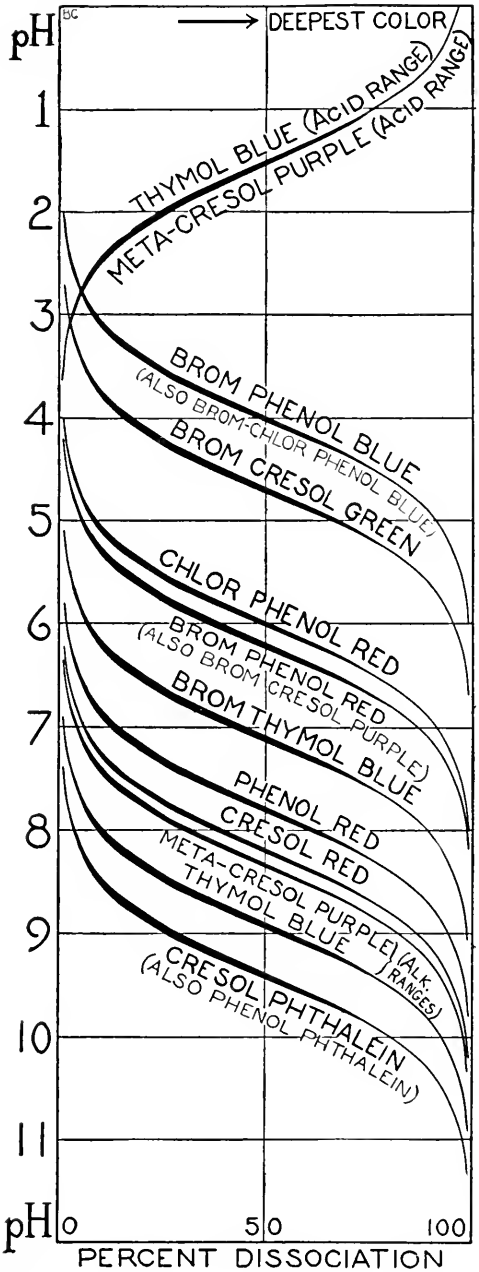


FIG. 1.—Ionization curves of some sulfonphthalein indicators, illustrating the general relationships among the acid-base indicators and the applications of equation 5.

Note: In some cases, the positions of the curves on the pH ordinate are approximate. Table 2 should be consulted for accurate values of pK'.

a full discussion of the properties and uses of pH indicators, see Clark (1928), and Kolthoff and Rosenblum (1937).

Within a short range on the pH scale on each side of the pK′ value, every color gradation of the indicator corresponds to a definite pH number; this zone may be called the sensitive range of the indicator. Throughout its sensitive range, an indicator can be used to determine the pH of a solution by comparing its color in the solution with that produced in standard solutions representing known pH numbers.

The indicators. A selection of indicators is presented in Table 2. All but three of the compounds are sulfonphthaleins which are particularly useful in bacteriological work because of their high tinctorial power, low or moderate salt and protein errors, and relative resistance to bacterial action. Table 2 gives the pK′ values of the indicators and their sensitive ranges. The last column, and footnote *b* of the table give specifications for the preparation of stock solutions of the mono-sodium salt of each of the sulfonphthaleins.

TABLE 2

ACID-BASE INDICATORS*

Name	pK′	pH-range and Colors	Recommended Conc. % (a)	Ml. of 0.01 M NaOH per 0.1 gm. (b)
Thymol blue (acid range)	1.7	Red 1.2–2.8 yellow	0.04	21.5
Methyl orange (c)	3.5	Red 3.1–4.4 yellow	0.05	(d)
Bromphenol blue	4.0	Yellow 3.1–4.7 blue	0.04	14.9
Bromcresol green	4.7	Yellow 3.8–5.4 blue	0.04	14.3
Methyl red	5.0	Red 4.2–6.3 yellow	0.02	(e)
Chlorophenol red	6.0	Yellow 5.1–6.7 red	0.04	23.6
Bromcresol purple	6.2	Yellow 5.4–7.0 purple	0.04	18.5
Bromthymol blue	7.1	Yellow 6.1–7.7 blue	0.04	16.0
Phenol red	7.8	Yellow 6.9–8.5 red	0.02	28.2
Cresol red	8.3	Yellow 7.4–9.0 red	0.02	26.2
Thymol blue (alk. range)	8.9	Yellow 8.0–9.6 blue	0.04	21.5
Phenolphthalein	9.7	Colorless 8.3–10.0 red	0.10	(f)

*See Clark (1948), and Kolthoff and Rosenblum (1937).

(a) Stock solutions in 95% ethanol for the indicator acids, or in water for the indicator salts, unless otherwise specified.

(b) Grind 100 mg. of the pure indicator acid with the amount of NaOH specified, and when solution is complete dilute with water to a volume that will yield the concentration recommended in column 4.

(c) Do not use with phthalate buffers.

(d) Dissolve 50 mg. in 100 ml. water.

(e) Dissolve 20 mg. in 60 ml. 95% ethanol, and add 40 ml. water.

(f) Dissolve 100 mg. in 65 ml. 95% ethanol, and add 35 ml. water.

It will be noted from footnote *a* that ethanolic solutions are ordinarily satisfactory. For precise work, however, aqueous solutions of the indicator salts are preferable to the alcoholic solutions of the free acids. To obviate the labor of preparing the neutralized solutions, some makers now offer the soluble salts of the sulfonphthaleins. They are ammonium, sodium, or possibly other salts of these compounds. In ordinary use, the indicator salts contribute negligibly to the total ions present in a test solution, and the nature of the cation may be of no consequence. However, in some studies

of bacterial nutrition, the kind of cation and even the small amounts thus added may be of significance. In such cases, it is advisable to learn from the maker what cations (Na, NH_4, etc.) are present in the indicator salt in order to make due allowance for their possible effects.

The colorimetric method of pH determination depends on matching the color of a suitable indicator in the unknown solution with that of the same indicator in a standard. The standards can be set up in two different ways: by means of buffer standards or by means of "drop-ratios". These will be considered in detail presently. In brief outline, the colorimetric method includes these major steps:

1. Selection of the appropriate indicator.
2. Preparation of color standards.
3. Color comparison for pH determination.

Later paragraphs will outline essential specifications that must be observed in each of these steps in order to assure reliable results.

Selection of the appropriate indicator. Test successive small portions (1 ml.) of the unknown with a drop of bromthymol blue (BTB). If the color produced is orange or red then the unknown is probably in the range of pH covered by thymol blue (acid range). If the BTB color is yellow, repeat the test with the indicators of successively lower pK′ (see Table 2) until that indicator is found which gives a color within its sensitive or useful range. If the BTB color is blue, proceed in like manner with indicators of higher pK′ until the appropriate indicator is found. Of course, if the unknown is more acid than pH 1 or more alkaline than pH 10, none of the indicators listed in Table 2 will serve.

If the unknown solution is unbuffered (*e.g.*, water or saline) or very weakly buffered, the buffering effect of the added indicator may prevail and significantly change the pH of the unknown. In such cases, special methods are required (see Clark, 1928).

It is plain that a rough idea can be obtained as to the pH value of a sufficiently buffered solution by simply finding which indicators give their acid color in it and which give their alkaline color. Indeed, the intelligent employment of indicators with overlapping pH ranges can be made to define the upper and lower limits of a relatively narrow zone of pH within which lies the pH of the solution under study (Small, 1946). Accuracy, however, can be obtained only by actual comparison with the colors produced by the indicators in solutions (buffers) whose pH values are known, or produced by application of equation 5 (drop-ratio method, p. 12-14).

Buffer solutions and color standards. A considerable variety of buffer solutions have been proposed; and many of them are discussed and described by Clark (1928). The compositions of the series of buffer standards proposed by Clark and Lubs (1917) are given in Table 3. Preparation of the stock solutions is described by Clark (1928).

After finding the appropriate indicator, prepare or select a series of properly graded standard buffer solutions sufficient in number to bracket the estimated pH of the unknown solution as determined in the preliminary trials. If, for example, the indicator selected is bromcresol green and the estimated pH of the unknown is near 6.0, then not more than five standards, namely buffers of pH 5.6, 5.8,

TABLE 3

COMPOSITION OF MIXTURES GIVING pH VALUES AT 20°C AT INTERVALS OF 0.2

From Clark (1928) p. 200–1.

KCl, HCl mixtures

pH	M/5 KCl	M/5 HCl	Dilute to
1.2	50 ml.	64.5 ml.	200 ml.
1.4	50 ml.	41.5 ml.	200 ml.
1.6	50 ml.	26.3 ml.	200 ml.
1.8	50 ml.	16.6 ml.	200 ml.
2.0	50 ml.	10.6 ml.	200 ml.
2.2	50 ml.	6.7 ml.	200 ml.

Phthalate, HCl mixtures

pH	M/5 KH Phthalate	M/5 HCl	Dilute to
2.2	50 ml.	46.70 ml.	200 ml.
2.4	50 ml.	39.50 ml.	200 ml.
2.6	50 ml.	32.95 ml.	200 ml.
2.8	50 ml.	26.42 ml.	200 ml.
3.0	50 ml.	20.32 ml.	200 ml.
3.2	50 ml.	14.70 ml.	200 ml.
3.4	50 ml.	9.90 ml.	200 ml.
3.6	50 ml.	5.97 ml.	200 ml.
3.8	50 ml.	2.63 ml.	200 ml.

Phthalate, NaOH mixtures

pH	M/5 KH Phthalate	M/5 NaOH	Dilute to
4.0	50 ml.	0.40 ml.	200 ml.
4.2	50 ml.	3.70 ml.	200 ml.
4.4	50 ml.	7.50 ml.	200 ml.
4.6	50 ml.	12.15 ml.	200 ml.
4.8	50 ml.	17.70 ml.	200 ml.
5.0	50 ml.	23.85 ml.	200 ml.
5.2	50 ml.	29.95 ml.	200 ml.
5.4	50 ml.	35.45 ml.	200 ml.
5.6	50 ml.	39.85 ml.	200 ml.
5.8	50 ml.	43.00 ml.	200 ml.
6.0	50 ml.	45.54 ml.	200 ml.
6.2	50 ml.	47.00 ml.	200 ml.

KH_2PO_4, NaOH mixtures

pH	M/5 KH_2PO_4	M/5 NaOH	Dilute to
5.8	50 ml.	3.72 ml.	200 ml.
6.0	50 ml.	5.70 ml.	200 ml.
6.2	50 ml.	8.60 ml.	200 ml.
6.4	50 ml.	12.60 ml.	200 ml.
6.6	50 ml.	17.80 ml.	200 ml.
6.8	50 ml.	23.65 ml.	200 ml.
7.0	50 ml.	29.63 ml.	200 ml.
7.2	50 ml.	35.00 ml.	200 ml.
7.4	50 ml.	39.50 ml.	200 ml.
7.6	50 ml.	42.80 ml.	200 ml.
7.8	50 ml.	45.20 ml.	200 ml.
8.0	50 ml.	46.80 ml.	200 ml.

TABLE 3—(*Continued*)

COMPOSITION OF MIXTURES GIVING pH VALUES AT 20°C AT INTERVALS OF 0.2.

From Clark (1928) p. 200–1

Boric acid, KCl, NaOH mixtures			
pH	M .5 H_3BO_3 M .5 KCl	M .5 NaOH	Dilute to
7.8	50 ml.	2.61 ml.	200 ml.
8.0	50 ml.	3.97 ml.	200 ml.
8.2	50 ml.	5.90 ml.	200 ml.
8.4	50 ml.	8.50 ml.	200 ml.
8.6	50 ml.	12.00 ml.	200 ml.
8.8	50 ml.	16.30 ml.	200 ml.
9.0	50 ml.	21.30 ml.	200 ml.
9.2	50 ml.	26.70 ml.	200 ml.
9.4	50 ml.	32.00 ml.	200 ml.
9.6	50 ml.	36.85 ml.	200 ml.
9.8	50 ml.	40.80 ml.	200 ml.
10.0	50 ml.	43.90 ml.	200 ml.

Notes. Overlapping members of the above series should be checked for consistency, i.e., phthalate "5.8" to "6.2" should match phosphates of the same pH numbers when tested with bromcresol purple; likewise for phosphate and borate "7.8" and "8.0" when tested with cresol red.

According to more recent assumptions used in standardization, the pH values given in the above table are too low by about 0.03 to 0.04 unit of pH.

6.0, 6.2 and 6.4, should suffice to safely bracket the actual pH of the unknown.

In preparing for the actual measurement, the unknown and the color standards should be contained in clear glass tubes selected for uniform bore, wall thickness, and inherent color. It is essential that the total concentration of indicator in the unknown be exactly the same as that in each of the color standards. This is best accomplished by accurately measuring, with a pipet, equal amounts of indicator (*e.g.*, 0.50 ml.) into equal amounts (*e.g.*, 10.0 ml.) of each of the selected standard buffer solutions. The indicator may be satisfactorily measured in drops provided the dropper tip is properly shaped (not too blunt), and the dropper is held vertically during the measurement. The use of excessive amounts of indicator may introduce difficulties; the minimum quantity necessary to produce recognizable coloration is desirable from the theoretical standpoint. It is essential, of course, that the indicator be uniformly distributed throughout the solutions to which it is added.

Prepared buffer standards can be obtained from supply houses, either as solutions or as powders or tablets to be dissolved as needed. They may also be obtained in sealed glass tubes containing the indicator. Such commercial color standards are convenient and satisfactory. They presuppose the use of comparable concentrations of indicator in the solution under test, and they must be used with the understanding that they are not permanent and may need to be checked or renewed at least once a year. All such indicator standards should be kept in the dark when not in use.

Color comparison. This procedure, commonly miscalled colorimetry, requires intelligent application to yield reliable results. The subject is adequately discussed by Clark (1928, 1948). Accurate color comparison of a standard solution with an unknown requires uniformity of the following conditions: the optical path (*i.e.*, distance through the solutions traversed by the light), transparency, wall thickness and color of the containers, concentration of indicator

in each of the solutions, and radiant power incident upon the systems under comparison. Also, any inherent color in the unknown must be compensated by an equivalent amount in the optical path through the standard. These conditions are met by selecting clear, unscratched tubes of uniform bore, glass thickness, and color, by having the same concentrations of indicator in the unknown and the standard, by dispersing the color uniformly in the solutions, and by employing proper illumination.

The color comparison is conveniently made in a comparator block of the type described by Clark (1928, 1948). Various forms of this are obtainable from supply houses. Two pairs of tubes are arranged in the comparator as follows: *1*, a tube containing buffer standard plus indicator behind which is placed a tube containing the unknown solution to compensate for inherent color, and *2*, a tube containing the unknown solution plus indicator backed by a tube containing distilled water. The two pairs of tubes are viewed against a uniform source of white light so placed that the beams incident upon the two systems are of the same radiant power. The color standards are successively compared with the unknown until a match is obtained, thereby establishing the pH of the unknown. If the color of the unknown falls between those of two adjacent standards an interpolated pH number may be estimated.

Systems of fixed or "permanent" color standards are also available. These standards consist of colored glasses or other transparent material. Since the spectral absorptions of such standards would hardly be expected to be exactly the same as those of the indicators that they are supposed to match, the applicability and accuracy of these fixed standards must be determined in each case before they are placed in service. Acceptable sets of such standards can be of great convenience in the bacteriological laboratory, especially for approximate determinations.

The drop-ratio standards of Gillespie. If commercial color standards are not available and there are no facilities for making standard buffer solutions, color standards may be prepared by the drop-ratio method as refined by Gillespie (1920). The method of preparing the standards consists in setting up pairs of tubes, containing stepwise proportions, of the full alkaline color and the full acid color of an indicator in such a manner that the resulting color of each pair, when properly viewed, represents a definite pH within the sensitive range of that indicator.

A general notion of the arrangement and composition of the drop-ratio color standards may be obtained from inspection of Table 4. The preparation of the standards is explained in the next two paragraphs and in Table 5.

Although the alcoholic solutions of the indicator acids mentioned in Table 2 may be used, Gillespie recommends for accurate work the use of aqueous solutions of the indicator salts (the preparation of which is specified in Table 2), except in the case of methyl red. Table 5, lower half, gives specifications for the recommended concentrations of seven of the indicator stock solutions. The exact concentration of the indicator solutions is not very significant in much bacteriological work.

Select 18 test tubes of approximately the same bore (between 12 and 15 mm.). They can be selected by adding 10.0 ml. of water to a large number of test tubes and choosing a lot in which the columns of water come to approximately the same height (*i.e.*, ± 1.5 mm.).

TABLE 4

DROP-RATIO COLOR STANDARDS FOR pH DETERMINATIONS

Tube pairs	Quantity of indicator solution to be added to each tube later to receive dilute alkali or acid and then brought to final volume of 5 ml.	
	Acid tubes	Alkali tubes
Pair No. 1	9 drops*	1 drop
Pair No. 2	8 drops	2 drops
Pair No. 3	7 drops	3 drops
Pair No. 4	6 drops	4 drops
Pair No. 5	5 drops	5 drops
Pair No. 6	4 drops	6 drops
Pair No. 7	3 drops	7 drops
Pair No. 8	2 drops	8 drops
Pair No. 9	1 drop	9 drops

*If a little more accuracy is desired one may use a 1 ml. pipet graduated in tenths and use the specified number of tenths of a milliliter instead of drops in preparing these tubes. In that case each tube should be brought up to a total volume of 10 ml. instead of 5 ml.

TABLE 5

DATA FOR DETERMINING pH VALUE BY THE DROP-RATIO METHOD

Pair	No. of drops of indicator		pH value represented by each pair of tubes						
	Alkali tube	Acid tube	Brom phenol blue	Methyl red	Brom cresol purple	Brom thymol blue	Phenol red	Cresol red	Thymol blue
1	1	9	3.0	4.05	5.2	6.15	6.85	7.35	7.95
2	2	8	3.4	4.4	5.6	6.5	7.2	7.7	8.3
3	3	7	3.6	4.6	5.8	6.7	7.4	7.9	8.5
4	4	6	3.8	4.8	6.0	6.9	7.6	8.1	8.7
5	5	5	4.0	5.0	6.2	7.1	7.8	8.3	8.9
6	6	4	4.2	5.2	6.4	7.3	8.0	8.5	9.1
7	7	3	4.4	5.4	6.6	7.5	8.2	8.7	9.3
8	8	2	4.6	5.6	6.8	7.7	8.4	8.9	9.5
9	9	1	4.9	5.95	7.0	8.05	8.75	9.25	9.85
Data as to stock solutions									
Percent concentration of indicator salt or acid.....			0.008 salt in water	0.008 acid in 95% alcohol	0.012 salt in water	0.008 salt in water	0.004 salt in water	0.008 salt in water	0.008 salt in water
Quantity N/20 NaOH to produce alkaline color*...			1 drop	1 drop	1 drop	1 drop	1 drop	1 drop	2 drops
Quantity of acid† to produce acid color			1 ml.	1 drop	1 drop	1 drop	1 drop	1 drop†	1 drop†

*If the standards are prepared by the method suggested in the footnote to Table 4 (i. e., measuring the indicator in tenths of 1 ml. and diluting to 10 ml.) it is well to use N/10 instead of N/20 NaOH to assure proper strength. The exact concentration or the exact number of drops used is of no great importance.

†Use approximately N/20 HCl (or N/10 if the method is modified as indicated in the footnote to Table 4) except in the case of cresol red and thymol blue. In the case of these two indicators a weaker acid must be used. Gillespie recommends 2 percent H_2KPO_4, or in the case of thymol blue no acid need be used, water alone having a sufficiently high pH value to bring out the full acid color.

Place these 18 tubes in two rows in a rack, 9 tubes in each row. To the left hand tube in the front row add 9 drops of the indicator solution, in the second tube place 8 drops, and so on to the last tube which should contain 1 drop. In the back row of tubes place 1 drop in the left hand tube, 2 in the next, etc., up to 9 in the last. Make up approximately N/20 stock solutions of NaOH and HCl (*i.e.*, 0.2% NaOH; and 1 ml. concentrated HCl (sp. gr. 1.19) diluted to 240 ml.). Then, except in the case of those indicators for which different directions are given in Table 5, add one drop of the stock acid solution to each tube in the front row and 1 drop of the stock alkali solution to each tube in the back row; add enough distilled water to each tube to bring its total contents to 5 ml., thoroughly mix the contents of each tube and return to its place in the rack. It will be seen from Table 5 that two of the indicators, namely thymol blue and bromphenol blue, require more of the alkali or the acid, respectively, than the other standards in order to insure the appearance of full alkaline or acid color. In the case of thymol blue (alkaline range) and cresol red, the production of the required acid color (yellow) requires not a strong acid but a weaker one such as mono-potassium phosphate or, in the case of thymol blue, distilled water alone.

The arrangement of tube-pairs indicated in Table 4 produces progressively different colors corresponding to steps of 10% in the transformation of the indicator from its acid to its alkaline color. That is, each pair of tubes, when aligned between the eye and a source of white light, will show a color mixture corresponding to a definite pH. This pH can be computed by means of equation 5 which can be rewritten as

$$\mathrm{pH} = \mathrm{pK}' + \log \frac{\text{drops of alkalinized indicator}}{\text{drops of acidified indicator}} \qquad (5a)$$

The fraction on the right side of the above equation is called the drop-ratio. The values of the standards for seven of the indicators are given in Table 5. They may be computed for the other indicators by using the above equation and the pK′ values in Table 2.

For approximate work it is often possible to compare the Gillespie standards with the unknown by merely holding the two tubes of the standard in the hand between the eye and a source of light. For accurate work, however, a comparator block must be used, but one with six holes instead of four, so that a tube of the unknown solution (without indicator) can stand behind the pair of tubes of the standard. The tube of the unknown for comparison with the standard should contain the same amount of indicator as the *sum* of those in the two standard tubes, *i.e.*, ten drops per 5 ml.; and, of course, this tube must be backed by two tubes of water to equalize the optical path through the standard pair.

Indicator Papers. Passing mention may be made of these laboratory aids for the approximate measurement of pH. Red and blue litmus papers for the detection of alkalinity and acidity are well known. Papers impregnated with other indicators, singly or in various combinations, can be made or obtained on the market. Those with a single indicator may be of use to detect roughly, (about ±0.3 to 0.4 pH), values within a relatively narrow zone of pH; those with indicator combinations enable one to

detect, more roughly, pH values over wider zones of pH. Such papers are more reliable in buffered solutions than in unbuffered ones.

To be emphasized, is the fact that the capillary action of the paper and of the sizing materials on the paper fibers may interfere, through selective sorption, with the normal interaction of solution and indicator. Generally speaking, a generous time of soaking of the paper for the establishment of equilibrium, seems desirable. On the other hand, a standardization of the procedure may permit a short exposure (30 sec.) to yield reproducible results, which are approximate in any case. See Kolthoff and Rosenblum (1937). Indicator papers are not recommended, except when the use of indicator solutions is precluded and a mere approximation is sufficient.

Titratable Acidity, Buffer Action, and pH Adjustment of Culture Media

In the titration of an acid with an alkali, or vice versa, a pH is reached at which the number of equivalents of acid equals the number of those of alkali. This pH is the *equivalence point* ("end-point").

If both the acid and the alkali are completely ionized, e.g., HCl and NaOH, it is simple to calculate that this pH is about 7, and that, in the case of 0.1 N reactants, the pH of the HCl solution will sweep precipitously from about pH 4 to 7 upon the addition of the last tenth per cent of NaOH; further, the addition of the first tenth per cent excess of NaOH will cause a shift from pH 7 to about 10. In other words, the titration curve, constructed by plotting pH as ordinates and per cent neutralization as abscissas, is very steep at the equivalence point (pH 7) in this titration.

The ideal indicator for the detection of this equivalence point would be one capable of giving a distinctive color at pH 7, *e.g.*, bromthymol blue. In practice, however, the steepness of the titration curve of the HCl at the equivalence point in the above example will permit this indicator to pass sharply from yellow to blue upon the final addition of a negligibly small excess of NaOH. For this reason, phenolphthalein (pK′ 9.7) is frequently used for this purpose because the first appearance of its pink color, at about pH 8.5, is a convenient and usually sufficiently accurate indication of the endpoint of such a titration.

In fact, except for refinements that may be neglected for ordinary purposes, pH 8.5, detectable by means of phenolphthalein, is a fairly satisfactory endpoint for the titration of strong acids and of all weak acids with pK′ values of less than 6.0. In the case of acids with pK′ values greater than 6.0, it is necessary, by application of equation 5, to calculate the pH of the equivalence point, and to refine the method of endpoint determination. For a discussion of the elementary theory of acid-base titration, see Clark (1928).

Titratable acidity of a culture. The titration of an acid (or a base) to an equivalence point, as discussed above, is a rational application of simple acid-base theory. On the other hand, in the titration of complex mixtures such as milk, tissue extract, or culture media, an equivalence point has no precise meaning. In such a case, the selection of an endpoint pH is arbitrary, and fixed by custom (*e.g.*, pH 8.5 with phenolphthalein) or by some special requirement.

In bacteriology, there is frequent need for determining the so-called titratable acidity produced during the growth of a culture in a fluid

medium. To do this, it is necessary first to select a baseline—that is, a pH number which is to be used as an endpoint in the titration and for the selection of an appropriate acid-base indicator. In the absence of special criteria, it is reasonable to choose as a baseline the pH of the uninoculated medium. The selection of pH 7 as a baseline may be acceptable, because many bacteria grow optimally in this region, not necessarily because it represents the pH of theoretical "neutrality". Other baselines may be chosen in accordance with the special requirements for which the titration is to be made.

The titratable acidity of the culture can be measured by titration of a known volume of the fluid with 0.1 *N* NaOH to the predetermined endpoint as shown by a standardized glass electrode or by the color of a suitable indicator. In the latter case, it is necessary to prepare for comparison an appropriate color standard representing the pH of the chosen endpoint (see earlier discussion of the essential requirements for adequate color comparison). If the endpoint pH is other than that of the uninoculated control, a titration is made of the latter and its titration value is subtracted algebraically as a correction or "blank", from that of the culture. The result is usually recorded as ml. of 0.1 normal acid per 100 ml. of the culture fluid. If the culture produces an alkaline reaction, the titration is performed with 0.1 *N* HCl, and recorded after correction, if any, in the same way but as a minus quantity of titratable acid. Special precautions are necessary if the titratable acidity is to include all of the volatile acids, including CO_2 and bicarbonate, that may be present in the culture that is being titrated.

It should be emphasized that, in most cases, the titratable acidity is merely a measure of the buffering capacity (see below) of the medium within the pH range observed. It does not permit further interpretation without additional data on the components of the culture. The titratable acidity is of some importance, along with final pH, in the comparison of high acid producing organisms. For such comparisons to be valid, it is necessary that the different organisms be grown in the same medium. Different media which vary in buffering capacity may yield misleading results.

Buffer action. The titration curve of a weak acid has a sigmoid shape, each end of the curve having a large (steep) slope, and the main central portion having a small slope. This small slope expresses the buffer action of the system, that is, the ability of the system (comprising the weak acid and its salt) to resist large change in pH on the addition of acid or alkali. The sigmoid shape of the titration curve expresses, therefore, the fact that the buffer action of such a system is maximal at the midpoint and decreases on either side of this point, first gradually and then more extensively as either end of the curve is approached. The limits of the pH zone of effective buffer action may be arbitrarily set at 1.5 pH units greater and less than the pK′ of the acid of the buffer system. It is obvious that increasing the concentration of the buffer system will increase its buffer action; therefore buffer action also depends upon the concentration of the buffer system.

The buffer action of a culture medium is dependent on its composition and may vary considerably in different regions of pH. Signifi-

cant results as to final pH and titratable acidity in cultures depend to a large extent on comparisons made in media having buffer action that is uniform and adjusted in amount to the purpose of the test. A method for estimating such buffer action is as follows:

Assume, for example, that the initial pH of a culture medium is 6.8 and that it is desired to measure the buffering capacity of the medium between the pH limits 5.0 and 8.0. This can be done by titrating an aliquot *e.g.*, 5 ml., of the medium with 0.05 N HCl to pH 5.0, and another aliquot with 0.05 N NaOH to pH 8.0. The sum of these titers gives a simple and useful measure of the buffering capacity of the medium within the pH zone 5.0 to 8.0. Brown (1921) has described the procedure and some of its practical uses.

The pH-adjustment of a culture medium. This is done with the medium at about 80 to 90% of its final volume. Prepare approximately normal NaOH and HCl stock solutions, and also, about 100 ml. of each of these solutions diluted with distilled water exactly to one-tenth concentration. Assume, for example, that the adjustment of a colorless medium is to be made to pH 7.0 before sterilization. Test the pH of the medium to establish whether acid or alkali will be required for adjustment to pH 7. To determine the amount required, titrate 5 ml. of the medium plus 5 drops of the appropriate indicator (*e.g.*, bromthymol blue) with the diluted acid or alkali until the color almost matches that of 10 ml. of standard buffer pH 7.0 plus 5 drops of the same indicator. Next, add water to the tube with medium to bring the volume to 10 ml., mix well, and make a proper comparison with the standard. If the color difference is small, then small additions of either acid or alkali may be made to bring about a correct match without changing significantly the necessary volume relations. If the color difference is large, the titration should be tried again. (In the case of a medium with inherent color, this should be compensated as previously described.)

From the titration value, a calculation can be made of the amount of the stronger acid or alkali to be added to bring the bulk of the medium to the desired pH. The pH of the medium is checked after the addition and, when correctly adjusted, the medium is diluted with distilled water to the final volume.

In making a colorimetric pH determination of a well-buffered medium that is already colored, it is permissible to dilute the test sample of the medium 1 to 5 or 1 to 10 with distilled water to thin out the inherent color before proceeding with the test. The change in pH due to such dilution of a well-buffered solution is usually negligible. On the other hand, caution must be observed in employing the dilution procedure on poorly buffered solutions, because the results may be misleading should the distilled water, or even the indicator solution, be too far from the desired pH.

The Measurement of Oxidation-Reduction Potentials

Introduction. The oxidation-reduction reaction

$$Cl_2 + 2I^- \longrightarrow 2\,Cl^- + I_2$$

represents an exchange of electrons between the chlorine:chloride system and the iodine:iodide system. These systems may be rep-

resented by the hypothetical "half-reactions"

$$Cl_2 + 2\epsilon \rightleftharpoons 2\ Cl^-$$

$$I_2 + 2\epsilon \rightleftharpoons 2\ I^-$$

to show the participation of electrons. In the interaction, chlorine is the electron-acceptor, and iodide the electron-donor.

The chlorine, the iodine and a considerable number of other systems can be studied by means of electric cells in which such systems can display their relative oxidation-reduction tendencies in terms of electrode potentials. The latter permit evaluation of the change in Gibbs free energy (see later) in the interaction of any two such oxidation-reduction systems.

Without going into details of derivation or refinements, we may state that the electrode equation for a reversible oxidation-reduction system has the general form:

$$E_h = E_o - \frac{RT}{nF} ln \frac{[\text{Reductant}]}{[\text{Oxidant}]} + \left(\begin{array}{l} \text{a function of pH and} \\ \text{dissociation constants} \end{array} \right) \qquad (6)$$

where E_h is the potential, in volts, referred to that of the normal hydrogen electrode; E_o is a constant characteristic of the system at pH 0; R is the gas constant, 8.315 volt-coulombs per degree per mole; T is the absolute temperature; n is the number of electrons involved in the oxidation-reduction process; F is the faraday (96500 coulombs); *ln* is the logarithm to the base e; and brackets represent concentrations of the reductant and oxidant. At any fixed pH, the first and last terms on the right side of the above equation may be combined as a constant, E'_o, then,

$$E_h = E'_o - \frac{RT}{nF} ln \frac{[\text{Reductant}]}{[\text{Oxidant}]} \qquad (7)$$

That is, $E_h = E'_o$ at any fixed pH when [Reductant] = [Oxidant].

It is apparent from equation 6, that the potential of such a system may be influenced by the pH of the solution; and the potential of one system may vary relative to that of another as the pH is varied. In fact, cases are known where system A can oxidize system B at one pH level, and system B oxidize system A at another. Hence the importance of comparing such potentials at the same pH, as well as the same temperature, and the desirability of specifying pH in connection with a statement of the E_h of a system.

Elaboration of the theory of reversible oxidation-reduction potentials can be found in Clark (1928, 1948), Clark, Cohen, *et al.* (1928), and modern texts on electrochemistry, such as Glasstone (1942).

There are two methods of measuring oxidation-reduction potentials, the potentiometric method and the colorimetric. Each has its advantages and disadvantages; but the potentiometric method is generally preferable for reasons that will appear below. In either case, it is usually necessary to deaerate the container and the solution to be measured by evacuation or by displacing gaseous and dissolved oxygen with an inert gas such as purified nitrogen. Deoxygenation is often accomplished spontaneously in the depths of an actively growing culture of facultative bacteria.

THE POTENTIOMETRIC METHOD

Electrode vessel. This may be a test tube with a constriction and bulb at its lower end or a more elaborate container depending on the requirements of the experiment. Such vessels are described by Clark, Cohen, *et al.* (1928), Borsook and Schott (1931), Allyn and Baldwin (1932), and Hewitt (1936).

Electrodes. A "noble" or "unattackable" metal is the electrode of choice. A coil of bright platinum wire has been frequently employed, but this is difficult to clean thoroughly and there is danger of entrapment of particulate material during a measurement. Platinum sheet, about 5 mm. square or larger, is preferable.

Gold-plated platinum electrodes seem to have certain advantages. They can be readily replated to provide a clean, new surface and thereby obviate erratic electrode behavior. Secondly, gold, being relatively impervious to hydrogen, should have less tendency to act as a hydrogen electrode in a culture producing appreciable quantities of molecular hydrogen. However, some observers do not consider this of much practical importance.

Electrodes should be checked for reliability by measuring the potential of a known oxidation-reduction system (*e.g.*, quinhydrone in 0.1 *M* HCl, $E_h = 0.6351$ at 25°, see p. 4.) Where possible, duplicate or multiple electrodes should be employed; and one that exhibits persistent erratic behavior should be discarded. Unless the solution or culture under examination is well stirred, the electrode reading may record merely a local oxidation-reduction potential rather than one representative of the solution as a whole. In a heavily growing culture, electrodes may become coated with adherent cell masses, and duplicate electrodes may show widely divergent potentials even when the culture is well stirred.

The common method of cleaning a platinum electrode involves cautious treatment with aqua regia, or hot concentrated nitric acid, or hot bichromate cleaning mixture, followed by thorough washing in water. For careful oxidation-reduction work, this procedure may not leave the metal surface altogether "inert". A more suitable procedure is to electrolyze a 1:1 solution of concentrated HCl with the electrode to be cleaned as the anode (gold-plated platinum may be deplated in the same way). The well washed electrode may be stored in distilled water. If the metal surface remains dry for any length of time, the electrode may be sluggish in reaching an equilibrium potential.

Calomel half-cell. (See also p. 3). The "saturated" type of any convenient form is generally suitable, preferably one that permits flushing of the siphon outlet with saturated KCl solution in order to wash away contaminations from liquid junction contacts. Liquid junction between the calomel half-cell and culture should be of a kind which can be made aseptically when desired. For ordinary purposes, this is conveniently accomplished by preparing a glass tube partly sealed at one end over a piece of acid-washed asbestos fiber. This tube is filled by means of a capillary-tipped pipet with melted KCl-agar (40 g. KCl per 100 ml. of 3% agar in water) and autoclaved. The partly sealed end of the tube is inserted into the culture to provide the "liquid" junction, and the open end is placed in bubble-free contact with saturated KCl solution leading to the calomel half-cell.

Potentiometer and galvanometer. Generally speaking, cell suspen-

sions and bacterial cultures are poorly stabilized with respect to oxidation-reduction potential. Consequently, disturbing polarization may occur if even the small amount of current necessary to operate the usual potentiometer and galvanometer circuit is allowed to pass through the half-cell containing the biological system under measurement; and the observed potential may be of uncertain accuracy and reliability. This difficulty can be minimized by the employment of a vacuum tube potentiometer-electrometer of the kind now in common use for glass electrode measurements and provided with a scale graduated in volts.

The oxidation-reduction cell is set up by joining the saturated calomel half-cell with the half-cell containing the solution or culture to be measured as indicated in the following scheme:

Pt; Solution X or PtAu; Culture X	KCl or KCl-agar bridge	Saturated calomel half-cell	(Pt)

Ordinarily, the potential of a culture is negative (reducing) to that of the calomel half-cell, and the metal terminals of the above oxidation-reduction cell are connected accordingly to the terminals of the potentiometer. The reading of potential thus obtained will be that referred to the calomel half-cell; and this observed potential, E_{obs}, can be converted to E_h, the potential referred to the standard normal hydrogen half-cell, by adding E_{obs} and E_{cal} algebraically. That is, $E_h = E_{obs} + E_{cal}$. Thus, if $E_{cal} = +0.250$ v. (see p. 3) and $E_{obs} = -0.150$ v., then $E_h = +0.100$ v.

Significance of E_h measurements. The potentiometric method is direct and relatively simple. The interpretation of the results is, however, another matter. Discounting subsidiary, but sometimes important, instrumental effects such as potentials due to liquid junctions, and temperature differences within the oxidation-reduction cell, all of which can be eliminated or minimized (see Clark, 1928), an observed E_h of a system such as ferric:ferrous iron, *under conditions of equilibrium and maximum work*, is a measure of the Gibbs free energy change, $nFE_h = -\triangle G$, in the reaction between the components of the two halves of the oxidation-reduction cell. This is the case for a considerable number of oxidation-reductions which, alone or in the presence of catalysts and mediators, can take place more or less rapidly and reversibly as if a transfer of electrons, with or without accompanying protons, were direct and complete. These are reactions between so-called electromotively active systems, the E_h of which is fixed, at constant pH, by a characteristic constant and by the relative concentrations (more accurately, activities) of the components of each such system. For example, a potential of the ferric:ferrous system in acid solution, can be defined by the relation:

$$E_h = E'_o - \frac{RT}{F} ln \frac{[Fe^{++}]}{[Fe^{+++}]} \qquad (8)$$

which implies the limitation that definite and significant potentials are possible only in the presence of *finite* ratios of oxidant to reductant. In addition, the total concentration of the reversible system may be decreased to and beyond a level at which traces of electromo-

tively-active contaminants attain dominance and an observed potential becomes unstable and difficult to interpret.

In contrast to the above mentioned reversible processes which are readily amenable to E_h measurement, there are a great many oxidation-reductions that proceed by a variety of mechanisms that do not permit formulation and precise measurement in terms of equilibrium states. Electrode potentials in such cases are difficult to interpret and of uncertain significance.

In cell suspensions and bacterial cultures, especially when deprived of free access of oxygen, there develops with time a progressively more negative potential which traverses the zones characteristic of reversible oxidation-reduction indicators (see next section). Polarization of the electrode or a small dose of an oxidant may reverse the trend of reduction potential temporarily, but the trend is resumed after a while to levels of potential that may sometimes be associated with the type of cell and the various metabolites in the suspension or culture. Duplicate electrodes in such systems may not be in good agreement at the start, but they will reach about the same limiting value in time. For examples, see Clark, Cohen, *et al.* (1928), Allyn and Baldwin (1932), and Hewitt (1936).

THE COLORIMETRIC METHOD

The empirical use of substances such as litmus or methylene blue as indicators of reduction in bacterial cultures is well known. For the determination of various degrees of reduction intensity an appropriate series of indicators is necessary. Among those available

TABLE 6

A SELECTION OF OXIDATION-REDUCTION INDICATORS

E'_o at pH 7, (30°)

(Values of E'_o between pH 5 and 9 will be found in Table 7)

	Compound	E'_o
a.	Phenol-*m*-sulfonate-indo-2,6-dibromophenol	0.273 v.
b.	*m*-Chlorophenol-indo-2,6-dichlorophenol	0.254
c.	*o*-Chlorophenol-indophenol	0.233
d.	2,6-Dichlorophenol-indophenol	0.217
e.	2,6-Dichlorophenol-indo-*o*-cresol	0.181
f.	1-Naphthol-2-sulfonate-indo-2,6-dichlorophenol	0.119
g.	Lauth's violet (Thionin)	0.062
h.	Cresyl blue	0.047
i.	Methylene blue	+0.011
j.	Indigo tetrasulfonate	−0.046
k.	Methyl Capri blue	−0.061
l.	Indigo trisulfonate	−0.081
m.	Indigo disulfonate	−0.125
n.	Gallophenine	−0.142*
o.	Brilliant alizarine blue	−0.173*
p.	Phenosafranine	−0.252
q.	Tetramethyl-phenosafranine	−0.273
r.	Safranin T	−0.285
s.	Induline scarlet	−0.299
t.	Neutral red†	−0.324
u.	Rosindone sulfonate No. 6	−0.385
	(Hydrogen at 1 atmosphere)	(−0.421)

*At 25°. †See footnote 3 in text.

TABLE

SELECTED OXIDATION-

Relation at

(Letters refer to compounds listed in

pH	a	b	c	d	e	f	g	h	i	j
5.0	+.390	+.391	—	+.366	+.335	+.262	+.138	+.149	+.101	+.065
5.5	.360	.359	—	.332	.300	.230	.109	.117	.072	.035
6.0	.330	.326	+.301	.295	.261	.196	.094	.189	.047	+.006
6.5	.301	.290	.269	.255	.220	.158	.077	.066	.028	−.022
7.0	.273	.254	.233	.217	.181	.119	.062	.047	+.011	−.046
7.5	.246	.220	.195	.182	.145	.080	.047	.030	−.005	−.066
8.0	.218	.188	.155	.150	.112	.046	.030	+.015	−.020	−.083
8.5	.192	.159	.117	.119	.081	+.016	+.017	−.001	−.035	−.099
9.0	.168	.133	.082	.089	.051	−.012	−.001	−.016	−.050	−.114

are reversible oxidation-reduction systems, the oxidants of which are usually colored and the reductants practically colorless. A number of such indicator systems have been characterized and may be employed, with due precautions, in determining an oxidation-reduction potential colorimetrically.

A selection of such indicators[3] is listed in Tables 6 and 7. Similar tabulations are given by Hewitt (1936). Fuller details can be found in Clark, Cohen, *et al.* (1928) and Cohen (1933, 1935). Table 6 gives the names of the indicators, listed in the order of their E'_0 values at pH 7.0; and Table 7 gives the corresponding E'_0 values at successive levels between pH 5.0 and 9.0. The magnitude of the salt and protein errors of these compounds has not been established.

Each indicator system listed in Tables 6 and 7 involves a two-electron transfer, and the relation of E'_0 to other factors *at fixed pH* is given by equation 9.

$$E_h = E'_0 - \frac{RT}{2F} ln \frac{[\text{Reductant}]}{[\text{Oxidant}]} \tag{9}$$

Converted to ordinary logarithms after insertion of numerical values, this equation becomes, at 30°C,

$$E_h = E'_0 - 0.030 \log \frac{[\text{Reductant}]}{[\text{Oxidant}]} \tag{10}$$

The relation of percentage reduction to potential as defined by the last term in equation 10 is given in Table 8. For example, if methylene blue is observed to be 80% reduced at pH 7, $E_h = 0.011 - 0.018 = -0.007$ volt.

[3]A special comment is necessary in regard to neutral red (compound *t* in Tables 6 and 7). It undergoes reversible reduction in the usual manner, and the colorless solution of reductant formed upon *rapid* reduction reoxidizes very rapidly when exposed to air. However, the reductant on standing in solution at pH 4 to 6 for a little time undergoes transformation to a fluorescent substance which is stable for days in the presence of air, but reoxidizes rapidly upon acidification. As an oxidation-reduction indicator, therefore, neutral red must be employed with due caution and can be used only for rough comparisons.

7

REDUCTION INDICATORS

E'_0 to pH (30°)

Table 6; the values listed are E'_0 in volts)

k	l	m	n*	o*	p	q	r	s	t†	u
+.038	+.032	−.010	−.003	−.040	−.158	−.157	−.197	−.235	−.205	—
+.006	−.002	−.040	−.042	−.080	−.188	−.194	−.227	−.253	−.236	—
−.021	−.028	−.069	−.077	−.112	−.215	−.225	−.251	−.268	−.265	−.298
−.043	−.056	−.098	−.110	−.142	−.234	−.252	−.270	−.284	−.294	−.349
−.061	−.081	−.125	−.142	−.173	−.252	−.273	−.285	−.299	−.324	−.385
−.077	−.103	−.148	−.172	−.203	−.269	−.288	−.300	−.314	−.352	−.425
−.093	−.121	−.167	−.202	−.226	−.284	−.303	−.316	−.329	−.382	−.460
−.108	−.137	−.184	−.232	−.251	−.299	−.319	−.331	−.344	−.410	−.491
−.123	−.152	−.199	−.262	−.279	−.314	−.334	−.347	−.359	−.438	−.520

*At 25°. †See footnote 3 in text.

Color standards. Since the compounds listed in Tables 6 and 7 are practically one-color oxidation-reduction indicators, color standards of sufficient approximation can be prepared simply by graded dilutions of the colored component, the oxidant. It should be borne in mind that some of the compounds are also acid-base indicators, therefore it may be necessary to set up the color standards in a buffer at the same pH as the solution or culture under test.

Colorimetric measurement. The general principles of color comparison, as outlined for the indicator method of pH-determination, are applicable here. In addition, special precautions are required to make certain that the measurement is a valid one. An indicator may fade in a test solution for reasons other than simple reduction. The compound may precipitate or adsorb on suspended particles, or it may be decomposed; in such cases judicious treatment with a suitable oxidizing agent (e.g., ferricyanide, or air) will not immediately restore the initial color of the oxidant. Moreover, many reversible oxidation-reduction systems are so sensitive to oxygen as to require extreme precaution for its exclusion. This applies to the electrometric method as well as to the colorimetric.

TABLE 8

Reduction	−0.03 log ratio	Reduction	−0.03 log ratio
%	Volts	%	Volts
1	+0.060	60	−0.005
10	0.029	70	−0.011
20	0.018	80	−0.018
30	0.011	90	−0.029
40	0.005	99	−0.060
50	0.000	100	(− ∞)

It is a fact that many biological systems act as if they contain, at any moment, only minute amounts of electromotively active oxidation-reduction substances, therefore the addition to such a system of even a small amount of indicator-oxidant may suffice to oxidize the system at once without appreciable reduction of the indi-

cator. This drawback cannot be overcome except by allowing sufficient time for the biological system to overcome the poising[4] effect of the added indicator. However, the time required may be very long (especially in relation to the most active period of a growing bacterial culture) so that it may be difficult or impossible to determine successive E_h values colorimetrically at brief intervals.

Furthermore, the indicator may not merely come into simple oxidation-reduction equilibrium with the components of the system under test. It may act catalytically to displace the oxidation-reduction equilibrium that it is supposed to measure; or it may be toxic toward living cells, or combine chemically with components of the system under test.

In summary, the indicator method, often applicable where it is impossible to employ an electrode, may give results that require considerable caution in interpretation, especially the results obtained on unstable oxidation-reduction systems or on biological material containing them.

REFERENCES

Allyn, W. P., and Baldwin, I. L. 1932. Oxidation-reduction potentials in relation to the growth of an aerobic form of bacteria. J. Bact., **23**, 369–398.

Borsook, H., and Schott, H. F. 1931. The rôle of the coenzyme in the succinate-enzyme-fumarate equilibrium. J. Biol. Chem., **92**, 535–557.

Brown, J. H. 1921. Hydrogen ions, titration and the buffer index of bacteriological media. J. Bact., **6**, 555–568.

Clark, W. M. 1928. The Determination of Hydrogen-Ions. *3rd Ed.* Williams and Wilkins, Baltimore.

Clark, W. M. 1948. Topics in Physical Chemistry. Williams and Wilkins, Baltimore.

Clark, W. M., Cohen, Barnett, *et al.* 1928. Studies on Oxidation-Reduction, I-X. Hygienic Laboratory Bulletin No. 151, U. S. Public Health Service, Washington.

Clark, W. M. and Lubs, H. A. 1917. The colorimetric determination of hydrogen-ion concentration. J. Bact., **2**, 1–34, 109–136, 191–236.

Cohen, Barnett. 1926. Indicator properties of some new sulfonphthaleins. Public Health Rpts., **41**, 3051–3074.

Cohen, Barnett. 1933. Reversible oxidation-reduction potentials in dye systems; (*also*) Reactions of oxidation-reduction indicators in biological material, and their interpretation. Cold Spring Harbor Symposia on Quantitative Biology, **1**, 195–204; 214–223.

Cohen, Barnett. 1935. Oxidations and Reductions. *Chapt. XIX in:* A Textbook of Biochemistry, by B. Harrow and C. P. Sherwin. W. B. Saunders Co., Phila.

Dole, M. 1941. The Glass Electrode. John Wiley and Sons, New York.

Gillespie, L. J. 1920. Colorimetric determination of hydrogen-ion concentration without buffer mixtures, with especial reference to soils. Soil Sci., **9**, 115–136.

Glasstone, Samuel. 1942. An Introduction to Electrochemistry. Van Nostrand, N. Y. See Chapt. VIII.

Hewitt, L. F. 1936. Oxidation-Reduction Potentials in Bacteriology and Biochemistry. *4th Ed.* London County Council.

Kolthoff, I. M., and Rosenblum, Charles. 1937. Acid-base Indicators. Macmillan, New York.

Small, James. 1946. pH and Plants. Van Nostrand, New York.

[4]Poising action of an oxidation-reduction system is analogous to buffer action of an acid-base system. (Compare paragraph on buffer action, p. 16.)

LEAFLET X

INOCULATIONS WITH BACTERIA CAUSING PLANT DISEASE

Prepared by

A. J. RIKER

Committeeman on Plant Pathological Methods

In collaboration with

P. A. ARK, CHARLOTTE ELLIOTT, and E. M. HILDEBRAND

3rd EDITION

Pure Culture Study of Bacteria, Vol. 13, No. 1-2

FEBRUARY, 1945

Note.—This leaflet is issued in accordance with the policy of including in the MANUAL material drawn up by committeemen or sub-committees who assume responsibility for methods outlined and opinions expressed. The committee will appreciate it if users of the MANUAL who have any fault to find with the methods or their presentation will communicate with the committee chairman or with the committeeman who has written this leaflet.

LEAFLET X

INOCULATIONS WITH BACTERIA CAUSING PLANT DISEASE

Introduction

The methods for studying the pathogenicity of bacteria in plants, and for making a few selected cognate investigations are briefly treated in this Leaflet. The procedures, in relation to handling certain organisms and to studying the diseases they induce, vary so widely that no given directions apply to the group as a whole. The selected representative methods included are thus to be considered primarily as guides to the beginner, and are to be modified as circumstances warrant.

Difficulty in interpretation is frequently encountered from variations in results, depending on the methods used. A given bacterial character may sometimes be positive when measured by one method and be negative when measured by a slightly different technic. Students should employ a known positive and a known negative as controls when making critical determinations. The method used should always be given or cited when a character is listed, so that the validity of the character can be correspondingly estimated by the reader. Some of the technical pitfalls to be avoided have been listed by Frobisher (1933).

A number of topics discussed in Leaflet VII regarding bacteria pathogenic on animals are applicable to bacteria pathogenic on plants. These include particularly: (1) identification of the active agent as the bacterial cell or its products; (2) distinction between invasion and the power to cause disease after entry; (3) variability in virulence of the pathogen, which requires single-cell cultures, and in susceptibility of the host, which frequently calls for plants with known genetic constitution, when critical studies are involved; and (4) relations between reactions induced in the test tube and in the host.

The pathogenicity of a microörganism may be proved by fulfilling Koch's postulates, which have been stated and modified in various ways, and which are so important that they are repeated here. One summarized statement follows: (1) The causal agent must be associated in every case with the disease, and conversely the disease must not appear without this agent. (2) The causal agent must be isolated in pure culture and its specific characters determined. (3) When the host is inoculated under favorable conditions with suitable controls, the characteristic symptoms of the disease must develop. (4) The causal agent must be reisolated, usually by means of the technic employed for the first isolation, and identified as that first isolated. Obviously, the demonstration of pathogenicity is made only after repeated trials, preferably with a number of different isolates which are of unquestioned purity. When the technic for cultivating causal

agents on artificial media has not yet been worked out, their pathogenicity is established in other ways (e. g., Rivers, 1937). When causal relations are being worked out, one may well differentiate between predisposing, inciting, and continuing causes. Various factors that influence the physiology of the plant may also affect pathogenicity.

The simpler methods for making isolations, for preparing and using both ordinary and differential media, and for studying the morphology and physiology of such bacteria have been adequately described in Leaflets II, IV, V, VI, and elsewhere (e. g., Rawlins, 1933; Riker and Riker, 1936; and Smith, 1905–1914, 1920). This Leaflet, therefore, is concerned primarily with methods of inoculation.

To insure against erroneous conclusions, the environmental conditions for experimental inoculations should be maintained as nearly as possible like those occurring in nature at the time of natural infection. When difficulty is experienced in artificial inoculation, careful, continued observation of the host plant at the time of natural infection may reveal the cause of the trouble.

In advanced research it appears that investigators working on pathogens, whether with plants, animals, or men, have many common interests. These include, for example, (1) life cycles, referring to changes in the morphology of individual cells and the relation of these different forms to virulence; (2) changes in colony characters and physiology, including particularly changes in pathogenicity; (3) factors attending changes, such as the time, frequency, and conditions of origin, as well as the influence of environment, and relations to earlier and succeeding generations; (4) statistical analyses to classify the origin and frequency of the variations observed; and (5) life histories of the pathogens in relation to entrance into the host, location, exit, and transmission to a new host, the well-known essentials of studies in epidemiology which are vitally influenced by variations in the pathogens.

Certain characteristics of plants not possessed by animals facilitate basic research on pathogenicity. Among the advantages in experimental work are the following: (1) Large numbers of hosts are easily available. The number used, whether 10 or 10,000, is selected on the basis of experimental needs. (2) The initial cost and expense of maintaining plants are relatively low. (3) The species of plants studied frequently contains varieties or selections possessing several degrees of resistance and susceptibility. (4) Plants are suited to a wide range of experimental procedures, such as regulation of internal temperature and moisture, that are not feasible with animals. (5) Epidemics[1] are induced with relative ease and without concern for the health of the technician or the public. (6) The genetic purity of the host can be assured. Seed from long lines of successively self-

[1] "Epidemic," in the original Greek meaning "on the people," was early applied to plant diseases, together with many other medical terms. It is an old and common word in plant pathology, although on etymological grounds its use for human disease alone is preferred by some medical authorities. In this paper, however, the broad definition from Gould's Medical Dictionary is followed, "Epidemic: of a disease affecting large numbers or spreading over a wide area."

fertilized parents is often available. When this is not sufficient, one can commonly find or develop experimental units all genetically identical through vegetative propagation. With such material any variations secured can be studied without concern that the host may have been obscuring pathogenicity. (7) Certain plant materials can be cultivated *in vitro* on media containing only nutrients for which the chemical formulae are known (reviewed by White, 1943).

Simple Representative Inoculation Methods

The actual method of making inoculations varies with circumstances. Some simpler methods are considered briefly by way of illustration. Methods for testing the relative efficiency of several techniques are considered in a later section.

SOIL "INOCULATION"

The introduction of large numbers of pathogenic bacteria into the soil depends upon growing sufficient quantities in cultures, either on agar or in liquid media. Special flasks, bottles, and other containers having adequate flat surfaces are employed. Most of the plant pathogens are aerobic and need to be incubated under pronounced aerobic conditions if the best growth is to be secured. When agar is used the surface growth on a suitable medium is washed or scraped off after sufficient growth has appeared, and a suspension is made. When a liquid medium is employed, a greater bacterial count per cubic centimeter is secured, with an organism like *Phytomonas tumefaciens* (Smith and Town.) Bergey *et al.*, by use of a medium less than 2 cm. deep or well aerated by some other means. Satisfactory aeration may be secured in deep liquid cultures by bubbling sterile air through a scintered glass or other aerator placed in the medium. In large containers aeration can be improved by a few pounds of pressure which forces more air to dissolve in the liquid. Maintaining such pressure also reduces contamination from leaky valves. Chemicals that poise the oxidation-reduction potential may be helpful. The highest count of active bacterial cells may be reached somewhat before the maximum turbidity is attained. Considerable turbidity is caused by bacterial gum. Usually the whole culture is employed for soil treatment; but one should avoid adding too much extraneous matter with the inoculum. Such aerated liquid cultures also work well with some fungi.

Soil may be "inoculated" by pouring liquid suspensions on relatively dry soil, allowing the water to be absorbed long enough to avoid puddling, and mixing. The quantity of culture used for each plant varies. One might begin with 1 part of culture to 10 parts of soil and use a handful of this mixture about the roots of each plant.

Inoculations through the soil are considerably more difficult than those with various other methods.

SEED INOCULATION

Perhaps the easiest way to infect a large population is through treatment of the seed. Legume root nodule bacteria from a fresh,

active culture grown on agar are shaken into a water suspension and are commonly spread on the seed just before planting. Many commercial "inoculations" are prepared by mixing the culture with some moisture absorbing powder, such as autoclaved ground peat. Wood flour is also quite absorbent, and contains almost no bacteria. If the seed is drill-sown, it is made only moist enough to distribute the bacteria well, and then dried sufficiently not to clog the drill. To secure uniform results it is best to use plenty of bacterial culture, for example, 5000 bacteria (plate count) per seed. For convenience in estimating the number of bacteria per seed a brief table is given by Fred, Baldwin and McCoy (1932), who review this general subject, showing the average number of seeds per pound of many legumes.

SPRAY INOCULATION

Spraying is one of the methods most commonly used in plant inoculation. It is particularly useful in diseases where the bacteria enter the host plants through natural openings such as stomata, water pores, and nectaries. For many simple tests, suspensions of bacteria are merely sprayed on the surfaces of susceptible leaves, stems, flowers, fruits, etc. For more exact tests, however, such as those for comparative virulence, it is common to suspend the growth from an agar culture in water, saline solution (0.9% NaCl), or a selected buffer (such as suitable mixtures of dilute K_2HPO_4 and KH_2PO_4), and to standardize the concentration according to a selected and measured turbidity. If the bacteria have been grown in liquid culture, the entire culture may be used. This procedure, however, is often unsatisfactory because, after spraying, secondary organisms may grow in the nutrient medium. It is frequently better to separate the bacteria from the medium by means of a centrifuge and to resuspend the cells as with the growth from agar media.

The number of bacteria in a suspension may be determined, for example, (1) by direct examination in a Petroff-Hausser counting chamber; or (2) by mixing a known volume of the bacteria with previously counted suspensions of yeast or red blood cells, making smears, and determining the relative number of bacteria and cells. Bacterial suspensions are often duplicated by comparing their turbidity with that of a graded series of barium sulfate standards (described by Riker and Riker, 1936). A common density for a bacterial suspension has the turbidity of a solution obtained by mixing 1 ml. of a 1% solution of barium chloride with 99 ml. of dilute sulfuric acid. Turbidity can be measured accurately and rapidly in an Evelyn densiometer.

The prepared bacterial suspension is filtered through cheesecloth, to remove small pieces of agar or other materials which might clog the spray nozzle, and is placed in the spraying device. The plants are sprayed so that good coverage is given especially to the lower sides of leaves which commonly have more stomata. The plants are placed in an environment where they will not dry off for a number of hours.

Certain additional precautions are sometimes necessary for best results, of which several are mentioned briefly. (1) The relative humidity of the air surrounding the host plant is maintained at satu-

ration before as well as after inoculation. The length of time varies with the host plant and the parasite. A saturated atmosphere for 6 to 36 hours in both instances favors infection with many leaf parasites. Various kinds of moist chambers, e. g., that described by Keitt *et al.* (1937), can be used in the greenhouse. Small outdoor plantings can be covered for a short time with a cloth tent (Keitt, 1918) and water sprayed over the exterior. The amount of moisture in the air apparently influences the inter-cellular humidity and, correspondingly, the susceptibility of the host. (2) If the plant parts are difficult to wet because of a waxy covering, the surface can be gently rubbed with a moist cloth. For work on a large scale, the suspension of the organism can be made in a solution of a spreader (e. g., castile soap, 1:1000) to reduce surface tension. The concentration is arbitrary and is varied according to requirements. Some spreaders, however, are toxic for certain pathogens. (3) A reduced oxygen supply may be important if the pathogen is a facultative anaerobe. For example, the protective wound-cork formation in potato tubers requires abundant oxygen, while certain bacterial pathogens, such as *Erwinia carotovora* (Jones) Holland, grow well with little oxygen. (4) Water pressure, suction, prolonged spraying, and other means can be used to saturate the intercellular spaces below the stomata and thus to improve the penetration of bacterial suspensions into these regions. This is particularly important with a pathogen, like that causing black fire of tobacco (Johnson, 1937), which is often not aggressive. With this method it is possible to induce necrotic areas on plants not ordinarily considered hosts of the microorganism used. Since bacteria that are usually considered as saprophytes have caused damage under these circumstances, care is necessary while interpreting such results. For example, such saprophytes would hardly fulfill the first of Koch's postulates, as given earlier.

WOUND INOCULATION

Suspensions of bacteria, small portions of culture, or of diseased tissue can be introduced into healthy plants through wounds when they do not readily gain entrance through natural openings or when heavier or more rapid infection is desired. The simplest procedure is to smear the point of a dissecting needle with the bacterial mass and to insert the needle into the plant tissue. If large numbers of inoculations are to be made, various instruments are useful. For example, an inoculator is described in detail by Ivanoff (1934). It consists of a hypodermic needle (size varied according to needs) with end closed and smooth-walled opening made on one side, of a suitable chamber to hold a bacterial suspension, and of a valve to regulate flow. This needle with a side opening may be used with an ordinary syringe. The common type of needle clogs too easily to be practical.

Known small numbers of bacteria may be introduced into microwounds by means of a micromanipulator. Such wounds may resemble those made by insects (Hildebrand, 1942).

INSECT INOCULATION

The translocation of microörganisms causing plant disease and their introduction into susceptible plants by insects is a large and relatively undeveloped field. The simplest technic with active insects like cucumber beetles or leaf hoppers is merely to place the plant to be inoculated in the same insect cage with an infested diseased plant. (Leach, 1940.)

For virus diseases, inoculation with slow-moving insects, like aphids, is accomplished by placing a paper on a caged plant to be inoculated, and by laying on this paper a portion of a diseased leaf which carries aphids. As the new leaf tissue dries, the insects crawl over the paper to the fresh leaf below. When insects are involved, a variety of special cages (Leach, 1940) may be used.

All stages in the life cycle of the insect employed must be considered because inoculation capabilities often vary in this respect. The insect should be identified by a competent authority, and if significant results are obtained a specimen should be deposited in a permanent reference collection.

A detailed discussion of methods for studying insect transmission has been given by Leach (1940). Some knowledge of the mouth parts of insects and of their feeding and breeding habits is necessary if insects are to be used successfully in inoculating bacterial plant pathogens. Insects are particularly important as carriers of virus diseases.

Before claims are made about insect transmission of a plant disease, demonstrations of the following (Leach, 1940) seem a minimum for proof: (1) close, but not necessarily constant association of the insect with diseased plants; (2) regular visits by the insect to healthy plants under conditions suitable for the transmission of disease; (3) presence of the pathogen or virus in or on the insect in nature or after visiting a diseased plant; (4) experimental production of the disease by insect visitation under controlled conditions and with adequate checks.

FUNGUS INOCULATION

In general, inoculations with the spores or mycelia of fungi differ only in detail from those made with bacteria. For pathogenic fungi, variations in the mode of entrance and in other important characters require modified procedures. Some of the more common methods are discussed by Riker and Riker (1936).

VIRUS INOCULATION

Brief mention is given to inoculations with viruses without implication that they are microörganisms. Experimental inoculations are more commonly accomplished by mechanical processes, insects (see Insect Inoculations), and grafting.

Mechanical inoculation of a virus is frequently made by grinding diseased tissue in a mortar with a little water and by rubbing the juice lightly over leaves of the host plant. With some viruses, the following modifications may be helpful. A favorable reaction between pH 7.0

and 8.5 may be obtained by placing a little M/10 K_2HPO_4 in the mortar before the leaves are triturated. Sometimes viruses have to be protected from rapid oxidation by means of 0.5% anhydrous Na_2SO_3. Just enough friction by a finger, cheesecloth, or similar agent is employed to injure the leaf hairs. With viruses difficult to transmit, better infection may be induced if a fine abrasive material (e. g., carborundom powder, 600 mesh) is lightly dusted on the leaf before it is rubbed. Some plant viruses are highly infectious. (Usually washing with soap and water is sufficient to remove infectious material from the technician's hands.) When the mechanical methods and insect vectors fail, two possibilities are left.

Budding or another form of grafting may be employed and is sometimes the only successful means of virus transmission. When grafts are made, special precautions must be taken to prevent desiccation of the grafted parts before union has been accomplished. This may be achieved by providing high air humidity, by suitable wrappers, or by spraying the scions with one of the commercial wax emulsions.

By means of dodder (Bennett, 1940) certain viruses not otherwise transmitted have been carried even from woody to herbaceous plants.

TREATMENT WITH BACTERIAL PRODUCTS

The metabolic products found in bacterial cultures are prepared and employed in a variety of ways which are not yet well worked out. Perhaps the least change occurs in the bacterial cells if they are centrifuged from a liquid culture and dried while frozen ("lyophile" apparatus described by Flosdorf and Mudd, 1935). The culture filtrate may be concentrated under reduced pressure at a little above room temperature and then "lyophilized" if desired.

A fermented culture or an aqueous extract may be sterilized and placed in a small container. If leaves with petioles or growing tops are removed from the host plant and are placed with the cut surfaces in such liquids, they commonly show injury within one day if much toxic material is present. Care is necessary while interpreting such injury because many constituents of media may be toxic, e.g., ammonia in alkaline material. Likewise, some non-parasitic as well as parasitic fungi produce toxic substances in culture that are not necessarily the reason for pathogenicity.

The metabolites are commonly applied either in liquid form or in a paste made with inert material, like lanolin or flour. The paste has the advantage of furnishing a continuous supply of material over a longer period with relatively less desiccation. It is commonly applied to a wound. The liquid can be introduced into the vascular system of a potted plant by placing cut roots extending from the base of the pot, or a cut petiole, into a container of the material. Likewise, a cup can be made from a rubber stopper and sealed on a plant stem with vaseline. The cup is filled with liquid, under which a cut is made into the vascular system, so that the liquid is taken by the plant directly into the transpiration stream. Or finally, the stem can be opened to form a small cavity which is kept filled by

means of a capillary tube and funnel. If an enzyme like pectinase is being tested, thin sections of tissue need merely be immersed in a few drops of the liquid.

So many substances appearing in cultures influence plants in one way or another that rigid controls are necessary in searching for the products responsible for pathogenicity. Whenever feasible, an attenuated culture of the same organism or a closely related nonpathogenic culture is carried in a parallel series of trials.

The methods of testing for plant "hormones" and "vitamines" are being revised so rapidly that an active investigator should be consulted for the latest procedure.

ANTIBODY PRODUCTION

Questions on the development of antibodies in plants following inoculation or natural infection are discussed in a considerable literature reviewed by Chester (1933). A number of controversial points are involved.

The injection of plant bacteria into an experimental animal (see Leaflet VIII) commonly results in the production of antibodies useful for various investigations. Serological work with plant pathogens is described by Link and his associates (1929, and earlier papers) and by various other investigators. Methods of applying the precipitin test to a study of certain viruses are given by Chester (1935).

Cognate Considerations

STRAIN VARIATIONS

When studies involving strain variations are made it is well to consider Frobisher's (1933) comment, "Plating and fishing of colonies, while generally useful, is not a sufficiently reliable method of purifying cultures in work involving bacterial variations. It is sometimes extremely difficult, if not impossible, to separate bacterial species by this means. Single-cell methods are much more reliable and, it would seem, furnish the only satisfactory means of solving our problems, but even such procedures as are at our disposal require very expert manipulation and may lead to error." The relative unreliability of the poured-plate technic for such studies has been discussed by Riker and Baldwin (1939). The need for cultures with a known origin from a single cell has stimulated much work on methods for securing them. Literature on this work has been reviewed by several writers, e. g., Hildebrand (1938). Unfortunately, several recent reports on bacterial variations have appeared in which the cultures were purified merely by several successive dilution plates, and such purified cultures were called "single-cell cultures." This misleading use of a well-established phrase provides both the investigator and the reader with a false sense of security.

Variations may be induced among plant pathogens by procedures very similar to those employed on other bacteria. Some of the considerations involved in such studies are discussed by Riker (1940). When there seems to be a bacteriophage in the complex, the general discussion by Krueger (1936) and the account by Thomas (1940) of a precursor may well be consulted.

The pathogenicity of crowngall bacteria can be destroyed (Van Lanen, Baldwin, and Riker, 1940) with certain amino acids and related compounds added to common media. Attenuation was commonly secured in 20 to 30 successive transfers. The rate of attenuation was increased if bacterial growth was reduced by the strength of the compound (e. g., 0.1 to 0.3% glycine) and by an alkaline reaction (e. g., pH 8.0).

The virulence of partly attenuated cultures was restored by long cultivation on suitable media and by ultra-violet irradiation (Duggar and Riker, 1940). Likewise, when a virulent culture was inoculated into a tomato stem above an attenuated culture, the gall about the attenuated culture was approximately as large as that about the virulent culture. A chemical gall served as well as that from a virulent culture (Riker, 1942).

PATHOGENS ACTING TOGETHER

Combinations of microörganisms sometimes induce symptoms different from those caused by any one alone. So long as the pathogens can be cultivated on artificial media, the principles in Koch's postulates can be applied with two or more causal agents. For example, a simple inoculation with one organism may involve a series of susceptible plants growing in a suitable environment with the living causal agent; and a parallel control series. With two causal agents, however, there should be four series of plants as follows: (1) with both living pathogens, (2) with only one living pathogen, (3) with only the other living pathogen, and (4) with neither living pathogen. Correspondingly, three causal agents would require eight series of plants.

CULTURES FROM ANOTHER LOCALITY

The use of a culture of a pathogen not already present on local plants requires critical consideration. The progress of bacteriology calls for reasonable freedom in the movement of cultures. This science, however, has a duty in the protection of local plant populations and requires that cultures or strains brought into a new locality should be handled with proper consideration of all the factors involved. While reasonable freedom in the shipment of such cultures from one laboratory to another is essential for certain work, it must be insisted that they be secured and studied only after both the investigators and their administrators have fully considered and accepted the responsibility involved. Younger research workers and particularly graduate students are advised to employ such cultures only after detailed plans have been made in conference with their advisors.

RELATIVE EFFICIENCY IN TECHNIC

The best methods of procedure for making inoculations and for recording results have not always been worked out and are not obvious from inspection. If the question is of sufficient importance, the answer may be secured statistically. There may be a question, with a leaf-spot organism, for example, as to whether it is better when making inoculations to spray or to make needle punctures. Likewise, when infection is secured, the question may occur whether the results should be recorded in terms of total number of lesions, of total tissue involved, the effect of the disease on yield, or of some other criterion. Such possibilities may be tested by means of the frequently described "analysis of variance" (e. g., Goulden, 1939). Thus the best method for making the trials and for recording the results may be determined. In general, the method that gives the greatest value for the variance ratio, "F", is the most desirable. This value indicates a greater uniformity in readings from different trials with the same technic, or a greater differentiation of the varieties used or treatments employed without a proportional increase in error.

ANTIBIOTICS

A recent survey (Osborn, 1943) has shown that various plants contain substances adversely affecting certain bacteria. Doubtless many instances (cf., Link and Walker, 1933; Ark and Hunt, 1941; Trussel and Sarles, 1943) occur in which various higher and lower forms of plant life contain chemicals that inhibit successful plant inoculations.

Records

Taking notes on plant inoculations presents various problems depending upon the experiment in hand. To assist with such records a tentative protocol (Table 1) has been prepared. For some lines of work it is obviously too complex while for others it is clearly inadequate.

A number of the items listed for records may be critical factors for the success or failure of an experiment. Since each one cannot be discussed, several examples are mentioned. (1) Infection may fail if the incubation temperature is either too low or too high. Many plant pathogens operate best between 18° and 30° C. (2) Plenty of moisture is important for disease development, a deficiency of water often being responsible for negative results. (3) The age of the plant or of the part inoculated may influence the result. The relatively young leaves are frequently more susceptible than old leaves to bacterial leaf spots. (4) Some varieties of plants are highly resistant to pathogens which readily attack other varieties. Similarly, different strains of bacteria often vary in pathogenicity.

TABLE 1. TENTATIVE PROTOCOL FOR PLANT INOCULATIONS

Host:
- Variety
- History
- Age
- Morphological condition
- Physiological condition
- Susceptibility
- Environment
- Treatment before
- Treatment after

Pathogen:
- Strain
- History
- Culture on
  - at. . . . . ° C.
  - for. . . . . days

Inoculum used:
- Diseased tissue
- Entire culture
- Bacteria:
  - Turbidity
  - Number per cc
- Filtrate
- Products
- Amount used per plant

Manner of inoculation:
- Through soil
- Through wounds
- By sprays
  - Spreader used
- By insects (name)
  - Stage in life cycle
- Other means

Incubation:
- Time
- Environment:
  - Temperature
  - Moisture
  - Light
  - Soil nutrients

Symptoms:
- Location
- Age of parts affected
- Severity
- Description:
  - Early
  - Medium
  - Final

Effect on yield:
- Quantity
- Quality

INTERPRETATION OF RESULTS

The results of research are valid only in accord with the reliability of the methods employed and the accuracy of their interpretation. After an experiment has been performed it is insisted that a report of such work must not be published for the use of others until repeated determinations have been made and the results have been satisfactorily analyzed. The simpler experiments are commonly performed with suitable controls at least in duplicate or triplicate, and carried through three separate times. A good investigator does not become so enthusiastic about an experiment that he fails to view it impartially and to accept sound evidence against it. On the contrary, he makes every reasonable effort before publishing to find an error in the experiment itself or in the conclusions drawn from it.

REFERENCES

ARK, P. A., and HUNT, M. J. 1941. Saprophytes antagonistic to phytopathogenic and other microorganisms. Science, **93**, 354.

BENNETT, C. W. 1940. Acquisition and transmission of viruses by dodder (*Cuscuta subinclusa*). Phytopath., **30**, 2 (Abstract).

CHESTER, K. S. 1933. The problem of acquired physiological immunity in plants. Quart. Rev. Biol., **8**, 129–54; 275–324.

————. 1935. Serological evidence in plant-virus classification. Phytopath., **25**, 686–701.

DUGGAR, B. M., and RIKER, A. J. 1940. The influence of ultraviolet irradiation on the pathogenicity of *Phytomonas tumefaciens*. (Abstract). Photopath., **30**, 6.

FLOSDORF, E. W., and MUDD, S. 1935. Procedure and apparatus for preservation in "lyophile" form of serum and other biological substances. J. Immun., **29**, 389–425.

FRED, E. B., BALDWIN, I. L., and MCCOY, E. 1932. Root Nodule Bacteria and Leguminous Plants. Univ. of Wis. Studies in Science No. 5.

FROBISHER, M. 1933. Some pitfalls in bacteriology. J. Bact., **25**, 565–71.

GOULDEN, C. H. 1939. Methods of Statistical Analysis. Wiley, New York and London.

HILDEBRAND, E. M. 1938. Techniques for the isolation of single microörganisms. Bot. Rev., **4**, 627–64.

————. 1942. A micrurgical study of crown gall infection in tomato. J. Agr. Res., **65**, 45–59, illus.

IVANOFF, S. S. 1934. A plant inoculator. Phytopath., **24**, 74–6.

JOHNSON, J. 1937. Relation of water-soaked tissues to infection by *Bacterium angulatum* and *Bact. tabacum* and other organisms. J. Agr. Res., **55**, 599–618.

KEITT, G. W. 1918. Inoculation experiments with species of Coccomyces from stone fruits. J. Agr. Res., **13**, 539–69.

KEITT, G. W., BLODGETT, E. C., WILSON, E. E., and MAGIE, R. O. 1937. The epidemiology and control of cherry leaf spot. Wis. Agr. Expt. Sta. Research Bul. No. 132.

KRUEGER, A. P. 1936. The nature of bacteriophage and its mode of action. Physiol. Rev., **16**, 129–72.

LEACH, J. G. 1940. Insect Transmission of Plant Diseases. McGraw-Hill, New York and London.

LINK, G. K. K., EDGECOMBE, A. E., and GODKIN, J. 1929. Further agglutination tests with phytopathogenic bacteria. Bot. Gaz., **87**, 531–47.

LINK, K. P., and WALKER, J. C. 1933. The isolation of catechol from pigmented onion scales and its significance in relation to disease resistance in onions. J. Biol. Chem., **100**, 379–383.

OSBORN, E. M. 1943. On the occurrence of antibacterial substances in green plants. Brit. J. Exp. Path., **24**, 227–231.

RAWLINS, T. E. 1933. Phytopathological and Botanical Research Methods. Wiley, New York and London.

RIKER, A. J. 1940. Bacteria pathogenic on plants. *In* The Genetics of Pathogenic Organisms, Pub. No. 12 of the A.A.A.S., Lancaster, Pa.

————. 1942. The relation of some chemical and physico-chemical factors to the initiation of pathological plant growth. Growth, Sup. to v. **6**, 4 Sym. Devlpmt. and Growth, 105–117, illus.

RIKER, A. J., and BALDWIN, I. L., 1939. The efficiency of the poured plate technic as applied to bacterial plant pathogens. Phytopath., **29**, 852–63.

RIKER, A. J., and RIKER, R. S. 1936. Introduction to Research on Plant Diseases. John S. Swift, St. Louis, Chicago, etc. [Planographed]

RIVERS, T. M. 1937. Viruses and Koch's postulates. J. Bact., **33**, 1–12.

SMITH, E. F. 1905–1914. Bacteria in Relation to Plant Disease. Vols. 1, 2, & 3. Carnegie Inst., Washington, D. C.

————. 1920. An Introduction to Bacterial Diseases of Plants. Saunders, Philadelphia and London.

THOMAS, R. C. 1940. Additional facts regarding bacteriophage lytic to *Aplanobacter Stewartii*. Phytopath., **30**, 602–611.

TRUSSEL, P. C., and SARLES, W. B. 1943. Effect of antibiotic substances upon Rhizobia. J. Bact., **45**, 29.

VAN LANEN, J. M., BALDWIN, I. L., and RIKER, A. J. 1940. Attenuation of cell-stimulating bacteria by specific amino acids. Science, 92, 512–513.
WHITE, P. R. 1943. A Handbook of Plant Tissue Culture. Jaques Cattell Press, Lancaster, Pennsylvania.

INDEX

TO

MANUAL

OF METHODS FOR

PURE CULTURE STUDY OF BACTERIA

EDITION OF 1948–49

ISSUED MAY, 1949

NOTE.—This index applies to the following editions of the various leaflets: Leaflet I, 9th, 1944; Leaflet II, 9th, 1944; Leaflet III, 4th, 1943; Leaflet IV, 9th, 1946; Leaflet V, 10th, 1947; Leaflet VI, 9th, 1942; Leaflet VII, 5th, 1948; Leaflet VIII, 6th, 1947; Leaflet IX, 10th, 1948; Leaflet X, 3rd, 1945.

INDEX[1]

[1] In this index Roman numerals refer to the leaflet number, inferior Arabic numerals to the year of the edition cited, and large Arabic numerals to the page of the leaflet.