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VOLUME VII

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## CONTENTS

### NO. 1, JANUARY, 1922

Certain Genera of the Clostridiaceae. Studies in Pathogenic Anaerobes. V. Hilda Hempl Heller.....	1
Studies upon Agglutination in the Colon-Typhoid Group of Bacilli. O. Ishii.	39
A Study of Spontaneous Agglutination in the Colon-Typhoid Group of Bacilli. O. Ishii.....	71
The Sources and Characteristics of the Bacteria in Decomposing Salmon. Albert C. Hunter.....	85
Viability of the Colon-Typhoid Group in Carbonated Water and Carbonated Beverages. S. A. Koser and W. W. Skinner.....	111
A Binocular Microscope Arranged for the Study of Colonies of Bacteria. Guilford B. Reed.....	121
An Investigation of American Stains. Report of Committee on Bacteri- ological Technic. Prepared by H. J. Conn, Chairman.....	127

### NO. 2, MARCH, 1922

Our Society. F. C. Harrison.....	149
Notes on the Gram Stain with Description of a New Method. Victor Burke.	159
Disinfection Studies. The Effects of Temperature and Hydrogen Ion Con- centration upon the Viability of <i>Bact. coli</i> and <i>Bact. typhosum</i> in Water. Barnett Cohen.....	183
Microorganisms Concerned in the Oxidation of Sulfur in the Soil. I. Intro- ductory. Selman A. Waksman.....	231
Microorganisms Concerned in the Oxidation of Sulfur in the Soil. II. <i>Thio- bacillus thiooxidans</i> , a New Sulfur-oxidizing Organism Isolated from the Soil. Selman A. Waksman and J. S. Joffe.....	239
The Production of Pink Sauerkraut by Yeasts. E. B. Fred and W. H. Peterson.....	257
Studies on the Biology of Lactic Acid Bacteria: a Summary of Personal Investigations. Costantino Gorini.....	271
A Method for the Cultivation of Anaerobes. L. D. Bushnell.....	277
Influence of Vacuum upon Growth of Some Aerobic Spore-Bearing Bacteria. L. D. Bushnell.....	283
Substitution of Brom-Thymol-Blue for Litmus in Routine Laboratory Work. H. R. Baker.....	301
The Use of Domestic Methylene Blue in Staining Milk by the Breed Method. W. A. Wall and A. H. Robertson.....	307

### NO. 3, MAY, 1922

Studies on Cultural Requirements of Bacteria. I. J. Howard Mueller.....	309
Studies on Cultural Requirements of Bacteria. II. J. Howard Mueller.....	325



A Method for Counting the Number of Fungi in the Soil. Selman A. Waksman.....	339
Studies on Thermophilic Bacteria. I. Aerobic Thermophilic Bacteria from Water. Lethe E. Morrison and Fred W. Tanner.....	343
An Apparatus for the Rapid Measurement of Surface Tension. Robert G. Green.....	367

## No. 4, JULY, 1922

Quantitative Determinations of Some of the Biochemical Changes Produced by a Saprophytic Anaerobe. L. D. Bushnell.....	373
The Proportion of Viable Bacteria in Young Cultures with Especial Reference to the Technique Employed in Counting. G. S. Wilson.....	405
A Method of Detecting Rennet Production by Bacteria. II. J. Conn.....	447

## No. 5, SEPTEMBER, 1922

The Relation of Vitamines to the Growth of a Streptococcus. S. Henry Ayers and Courtland S. Mudge.....	449
Salt Effects in Bacterial Growth. II. The Growth of <i>Bact. Coli</i> in Relation to H-Ion Concentration. James M. Sherman and George E. Holm....	465
A Note on the Morphology of Bacteria Symbiotic in the Tissues of Higher Organisms. Ivan E. Wallin.....	471
Observations on the Properties of Bacteriolysants (D'Herelle's Phenomenon, Bacteriophage, Bacteriolytic Agent, etc.). Part I. Wilburt C. Davison.	475
Observations on the Nature of Bacteriolysants (D'Herelle's Phenomenon, Bacteriophage, Bacteriolytic Agent, etc.). Part II. Wilburt C. Davison.....	491
<i>Clostridium Putrificum</i> ( <i>B. Putrificus</i> Bienstock), A Distinct Species. George F. Reddish and Leo F. Rettger.....	505
Transparent Milk as a Bacteriological Medium. J. Howard Brown and Paul E. Howe.....	511
The Use of Agar Slants in Detecting Ammonia Production and its Relation to the Reduction of Nitrates. G. J. Hucker and W. A. Wall.....	515
Methods of Pure Culture Study. Report of Committee on Bacteriological Technic. H. J. Conn, chairman.....	519
An Investigation of American Gentian Violets. Report of Committee on Bacteriological Technic. Prepared by H. J. Conn, Chairman.....	529

## No. 6, NOVEMBER, 1922

Method for the Isolation of Bacteria in Pure Culture from Single Cells and Procedure for the Direct Tracing of Bacterial Growth on a Solid Medium. J. Ørskov.....	537
Bacterial Autolysis. W. S. Sturges and L. F. Rettger.....	551
<i>Bacillus Hemoglobinophilus canis</i> (Friedberger) ( <i>Hemophilus canis emend</i> ). T. M. Rivers.....	579
Salt Effects in Bacterial Growth. III. Salt Effects in Relation to the Lag Period and Velocity of Growth. J. M. Sherman, G. E. Holm and W. R. Albus.....	583

CONTENTS

v

Further Observations on the "Color Standards" for the Colorimetric Determination of H-Ion Concentration. L. S. Medalia.....	589
The Cause of Explosion in Chocolate Candies. John Weinzirl.....	599
Microorganisms Concerned in the Oxidation of Sulfur in the Soil. IV. A Solid Medium for the Isolation and Cultivation of <i>Thiobacillus thiooxidans</i> . Selman A. Waksman.....	605
Microorganisms Concerned in the Oxidation of Sulfur in the Soil. V. Bacteria Oxidizing Sulfur under Acid and Alkaline Conditions. Selman A. Waksman.....	609





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OFFICIAL ORGAN OF THE SOCIETY OF AMERICAN  
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JANUARY, 1922

EDITOR-IN-CHIEF

C.-E. A. WINSLOW



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## CONTENTS

HILDA HEMPEL HELLER. Certain Genera of the Clostridiaceae. Studies in Pathogenic Anaerobes. V .....	1
O. ISHII. Studies upon Agglutination in the Colon-Typhoid Group of Bacilli.....	9
O. ISHII. A Study of Spontaneous Agglutination in the Colon-Typhoid Group of Bacilli.....	71
ALBERT C. HUNTER. The Sources and Characteristics of the Bacteria in Decomposing Salmon.....	85
S. A. KOSER AND W. W. SKINNER. Viability of the Colon-Typhoid Group in aerobated Water and Carbonated Beverages.....	111
GUILFORD B. REED. A Binocular Microscope Arranged for the Study of Colonies of Bacteria.....	121
An Investigation of American Stains.....	125

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## ERRATUM

*Journal of Bacteriology, Vol. VI, No. 6, November, 1921.* Line 17 from the top of page 560 reads "from pH to 8.0." It should read "from pH 5.0 to 8.0."





# CERTAIN GENERA OF THE CLOSTRIDIACEAE

## STUDIES IN PATHOGENIC ANAEROBES. V

HILDA HEMPL HELLER

*From the George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco*

Received for publication March 1, 1921

In a previous paper a classification was suggested for the group of anaerobic rods which include, roughly, the anaerobic members of the genus *Bacillus* of former workers. A family was proposed for these organisms, with the name *Clostridiaceae*,<sup>1</sup> and it was divided into two subfamilies, the *Putrificoideae*<sup>2</sup> or proteolytic anaerobes, and the *Clostridioideae*<sup>2</sup> or non-proteolytic anaerobes. Certain genera for these groups are proposed in the present communication. These in some cases unite various described species, while in other cases the genera themselves correspond to the former idea of species.

A key to the genera is also given, which is based on the action of the anaerobes on meat medium and on their general cultural behavior and morphology. Possibly the main lines for tribal organization will to some extent follow this key, but it pretends to be no more than an artificial arrangement. It is in no way complete and cannot be implicitly relied upon for purposes of classification. It is meant more as an index to the forms whose descriptions are sufficiently clear to warrant assigning them definite positions. The key will serve, for a time, as a nest of pigeonholes in which to place new species until material is sufficient for a complete reorganization, but workers should not try to place all newly discovered organisms in these genera.

The conservative worker, familiar with aerobic pathogens, who enters the anaerobic field, is all too prone to wish to "identify"

<sup>1</sup> For definition see Jour. Bact., 6, 536.

<sup>2</sup> For definition see Jour. Bact., 6, 550.

his anaerobic strains. A natural feeling exists among systematists, that the description of a new-found type as a new species is to be avoided unless the describer is very sure that his type differs from all others. There is a fear that a description carefully made will some day be discarded by a future taxonomist who finds it identical with a former description. This logical attitude has bred and fostered a wholly unscientific mania for identification of all new strains with organisms already described by others. In the case of parasites or in well-known groups this procedure will only occasionally lead the worker astray. In the anaerobic field it will do so frequently. The scientific attitude relative to the taxonomic affinities of anaerobic bacilli is to state the group or generic relationships of the organisms and then to describe them minutely. The routine bacteriologist should be content to assign an organism to its proper genus.

Colony formation in deep agar is included in the descriptions of genera, but a restriction of the genera to such types of colony form as are mentioned would be unwise. The definition of the Gram staining reactions should not be regarded as of great value in these descriptions, as the staining reactions of the organisms and the technique of workers are too variable. Sugar fermentations may be relied upon in the case of actively growing species only, and are valid only for media on which the behavior of a strain is constant. The constancy of the fermentations here quoted has not been verified by myself. Pathogenicity is not to be taken as a criterion for admission to a genus.

It is to be feared that bacteriological systematists who are unacquainted with the anaerobic group will object to the creation of so many genera as are here proposed. The Committee on Characterization and Classification of Bacterial Types (1920) propose but 38 genera for the orders *Actinomycetales* and *Eubacteriales*. The influence which the medical history of the science of bacteriology has had on this classification is marked. Two-thirds of the 38 genera recognized contain parasites. If we regard the anaerobic bacteria as wild plants growing in soil, as we have every reason for doing because of their many species, we

must classify them as do the botanists and not as do the bacteriologists. The family of *Orchideae* has 334 genera, the *Gramineae* 298, the *Rubiaceae* 377, the *Leguminosae* 399, and the *Compositae* 766 (Bentham and Hooker). The following classification imitates the taxonomic arrangement of botanical classifications in preference to bacteriological. It must be borne in mind that the date 1920 in bacteriologic systematics corresponds roughly to the date 1760 in botanical systematics.

Relative to the evolutionary history of the anaerobes we know nothing, but conjectures are not entirely out of place. Proteins and carbohydrates have existed together and have been destroyed by bacterial action since a remote geologic epoch. We cannot tell whether the organic catalyzers of one type of substance antedated those of the other or whether they are closely related. Botanists are not agreed as to what form of life first tenanted the globe. When looking for ancestral forms it is natural to seek in a group of organisms of great catalytic activity like the *Clostridiaceae*, for types which may synthesize simple substances, and if such are discovered it is not out of place to regard them as more closely related to the hypothetical ancestors of the group than those that do not synthesize inorganic substances. Such reasoning is of course entirely dependent on the homogeneity of the group under discussion. We have no means of proving the homogeneity of the *Clostridiaceae*. But when classified according to their chemical activities these organisms form a remarkable chain whose links we are all but warranted in regarding as the varied end points of an evolutionary process. As our most primitive type we may well choose the nitrogen-fixing anaerobes of the soil. It is to Winogradsky that we turn for the first demonstration of such organisms. He named his anaerobic nitrogen-fixing organism *Clostridium Pastorianum*. Bredemann declares that nitrogen-fixation is a variable power among all organisms of the amylobacter type, and he includes under the name amylobacter, *Clostridium Pastorianum* of which bacillus he possesses a strain. These organisms are highly saccharolytic, being pectin fermenters, and they do not split gelatin. Farther up the scale we find anaerobic rods which

are actively saccharolytic and do split gelatin, for example the Welch bacillus, vibron septique, and many other forms; these organisms do not liquefy coagulated serum or produce hydrogen sulfide or other protein split products in any considerable quantity. Most of them possess no diastatic power toward pentosans, and many fail even to split pentoses. The group next in the scale are those anaerobes which, though they fail to disintegrate coagulated serum and muscle particles, are sufficiently proteolytic to free considerable quantities of hydrogen sulfide in media rich in sulfur, such as blood broth. The oedematiens type, and other less well-known organisms may be placed here. Some of these are strongly saccharolytic and others are weakly so. Further advances in attack on the protein molecule are almost invariably accompanied by a decrease in saccharolytic power. *B. aerofectidus*, and the two strains which I term *Regillus* are apparently slightly proteolytic, and only moderately saccharolytic. The more highly proteolytic organisms usually split monohexoses, glucose only, or no sugars at all. The most highly adapted catalytic anaerobes are those that produce both acid and alkali from their substratum in sufficient quantities to keep their hydrogen-ion end point within their optimum range and which are so highly proteolytic that they disintegrate a great variety of protein molecules and split-products. Such types are *B. histolyticus* and *B. botulinus*. Another type, known as *Bifermentans*, keeps its end point within its optimum range of growth but is not sufficiently proteolytic to continue multiplication for a long period. Such organisms are not highly but widely specialized, and are adapted to fend for themselves because they are saccharolytic as well as proteolytic.

It is to render possible a future scientific and logical classification of anaerobic organisms that this key and these generic definitions are proposed. They are manifestly incomplete, and it is certain that careful taxonomic investigations will require the emendation of many of the proposed genera. But the present paper aims to blaze a trail where meandering paths have wandered—a trail upon which a highroad may be built in the future.

I wish most heartily to thank Dr. Karl F. Meyer for many helpful suggestions which he has given me during the compilation of this classification.

## ARTIFICIAL KEY TO THE GENERA OF THE CLOSTRIDIOIDEAE

- A. Do not produce H<sub>2</sub>S demonstrable by lead-acetate-paper test when grown in blood-broth.
- B. Do not liquefy gelatin
- C. Very large rods that form oval spores and store up starch in carbohydrate media.
- Genus 1. *Clostridium* Prazmowski emend. Heller. Type species *butyricum* as described by Winogradsky (1895).
- CC. Rods with spherical spores.
- D. Do not grow on media containing much protein.
- Genus 2. *Omeliaskillus* nov. gen. Type species *hydrogenicus* as described by Omeliansky (1895 and 1904b).
- DD. Grow on ordinary anaerobe media.
- E. Sides of the bacilli parallel, spores strictly terminal.
- Genus 3. *Macintoshillus* nov. gen. Type species *tetanomorphus* (pseudotetanus bacillus, McIntosh (p. 32), *Bacillus tetanomorphus* Committee (p. 41)), as described by the Committee.
- EE. Spores not always strictly terminal, sides of bacilli may not be parallel.
- Genus 4. *Douglasillus* nov. gen. Type species *sphenoides* (*Bacillus sphenoides* Douglas Fleming and Colebrook), as described by the Committee (p. 43).
- CCC. Slender rods with oval endspores, usually Gram-negative.
- D. Clot milk and attack various sugars, produce much acid.
- Genus 5. *Henryillus* nov. gen. Type species *tertius* (*Bacillus tertius* Henry) as described by Henry.
- DD. Do not clot milk, attack few or no sugars, produce little acid.
- Genus 6. *Flemingillus* nov. gen. Type species *cochlearius* (*Bacillus cochlearius* Douglas Fleming and Colebrook) as described by the Committee (p. 40).
- CCCC. Gram-positive rods which are not markedly slender and which produce oval spores.
- D. Clot milk, saccharolytic.
- E. Sporulate meagerly, attack a few sugars; occasionally moderately pathogenic tissue invaders.

Genus 7. *Vallorillus*, nov. gen. Type species *fallax* (*Bacillus fallax* Weinberg and Séguin) as described by the Committee (p. 27).

EE. Sporulate readily, attack several sugars; not known to be pathogenic.

Genus 8. *Multifermentans* nov. gen. Type species *tenalbus* (*Bacillus multifermentans tenalbus* Stoddard) as described by Stoddard (1919 b).

DD. Do not clot milk. Large Gram-positive rods with long ellipsoid spores.

Genus 9. *Hiblerillus* nov. gen. Type species *sextus* (bacillus VI of von Hibler) as described by von Hibler (1908). (Résumé by Weinberg and Séguin (p. 202).)

BB. Liquefy gelatin.

C. Produce stormy fermentation of milk and sporulate on alkaline media only.

Genus 10. *Welchillus* nov. gen. Type species *acrogenes* (*Bacillus acrogenes capsulatus* Welch and Nuttall), type 1 as defined by Simonds (1915 a and b).

CC. Do not produce stormy fermentation of milk.

D. Do not sporulate.

Genus 11. *Stoddardillus* nov. gen. Type species *egens* (*Bacillus egens* Stoddard) as described by Stoddard (1919 a).

DD. Sporulate readily.

E. Gram-positive, form woolly colonies in deep agar. Typically highly pathogenic tissue invaders of many species of animals.

Genus 12. *Rivoltillus* nov. gen. Type species *vibron* (the vibron septique of Pasteur) as defined in a future paper.

EE. Gram-negative, may contain Gram-positive granules. Form smooth lenticular or modified lenticular colonies in deep agar. Typically pathogenic for cattle, sheep, and guinea-pigs.

Genus 13. *Arloingillus* nov. gen. Type species *Chawoei* (*Bacterium Chawoei* Arloing, Cornevin and Thomas) as described in a future paper.

AA. Produce H<sub>2</sub>S demonstrable by a lead-acetate-paper test when grown in blood broth.

B. Produce a large amount of gas from carbohydrates. Heavy Gram-positive rods with little or no tendency to sporulation.



Genus 14. *Meycrillus* nov. gen. Type species *sadowa* nov. sp. to be described in a future paper.

BB. Produce less gas from carbohydrates. Heavy Gram-positive or Gram-negative rods that form oval spores that are usually subterminal.

Genus 15. *Navillus* nov. gen. Type species *maligni* (*Bacillus oedematis maligni* H. Novy) as described by Novy.

#### ARTIFICIAL KEY TO THE GENERA OF THE PUTRIFICOIDAE

A. Produce an alkaline reaction in meat medium but do not grossly disintegrate the particles of meat.

B. Minute Gram-positive rods which form few spores. Clot milk. Weak tissue invaders, or non-pathogenic.

Genus 16. *Seguinillus* nov. gen. Type species *acrofoetidus* (*Bacillus acrofoetidus* Weinberg and Séguin) as described by Weinberg and Séguin (p. 161).

BB. Gram-positive rods with oval subterminal or median spores that do not greatly distend the sides of the bacilli. Do not ferment milk. Typically pathogenic tissue-invaders.

Genus 17. *Regillus* nov. gen. Type species *progrediens* nov. sp. to be described in a future paper.

AA. Attack proteins somewhat more energetically than the above and produce a terra cotta coloration of the meat particles and soften and partially disintegrate them. (The terra-cotta color is not to be confused with the pink color produced in meat medium by the acid from sugar fermentation.) These organisms continue to multiply in meat medium at a moderate rate for months.

B. Show no blackening of the meat-particles on prolonged incubation.\* Gram-negative or weakly Gram-positive rods with oval subterminal or median spores that distend the bacilli.

Genus 18. *Robertsonillus* nov. gen. Type species *primus* (*Bacillus* I. Hempl) as described by Hempl.

BB. Show after approximately two weeks' incubation a blackening of some of the meat-particles. Gram-negative rods with spherical end-spores. Typically produce a neuro-toxin.

Genus 19. *Nicollaierillus* nov. gen. Type species *tetani* (*Bacillus tetani* Nicollaiier) to be described in a future paper.

---

\* "Incubation" refers to anaerobic incubation at 37°C except in the case of deep agar tubes which are incubated in air at 37°C. Meat medium is pH 7.2 to start with.

AAA. Highly proteolytic on meat medium for a short period. Produce a gray coloration or a slight blackening of the medium. Sporulate at an early stage and after three days' incubation vegetate very slowly indeed without so digesting the meat particles that they greatly decrease in size.

B. Thick rods with oval or oblong spores which are usually central and do not greatly distend the bacillus.

C. Resistant saprophytes. Occasionally invade tissue in company with other organisms or alone in debilitated individuals.

Genus 20. *Martellillus* nov. gen. Type species *bifermantans* (*Bacillus bifermantans sporogenes* Tissier and Martelly) as described by Tissier and Martelly.

CC. Delicate parasites. Gram-negative tendency.

Genus 21. *Recordillus* nov. gen. Type species *fragilis* nov. sp.

AAAA. Organisms highly proteolytic, producing on three days' incubation in meat medium partial destruction of the meat particles which continues on further incubation till the meat particles have greatly diminished in bulk. Coloration of meat usually dark brown or terra-cotta; blackening may or may not take place.

B. Slender rods with terminal oval spores.

C. Split sugars.

Genus 22. *Tissierillus* nov. gen. Type species *paraputrificus* (*Bacillus paraputrificus* defined by Bienstock 1906) as described by McIntosh under the name of *Bacillus putrificus* (p. 39).

CC. Do not split sugars.

Genus 23. *Putrificus* nov. gen. Type species *Bienstocki* (*Bacillus putrificus* Bienstock) as defined by Bienstock (1906).

BB. Heavy rods with subterminal or median oval spores.

C. Do not produce balls of amino-acid crystals to a striking degree.

D. Not so highly proteolytic as organisms of following groups. Meat particles not much decreased in size, spores often larger, and more nearly spherical than in following genera. Isolated colonies in deep agar usually, but not always, large, smooth lenticular or modified lenticular structures that do not become woolly. Typically produce a powerful neuro-toxin.

Genus 24. *Ermengemillus*, nov. gen. Type species *botulinus* (*Bacillus botulinus* van Ermengem) as described by Meyer and co-workers in a future paper.

DD. Highly proteolytic, blacken meat readily, produce penetrating foul odor. Form in deep agar large woolly or spherical colonies or lenticular colonies that show a tendency to become woolly. Are not pathogenic in pure culture.

Genus 25. *Metchnikovillus* nov. gen. Type species *sporogenes* (*Bacillus sporogenes* Metchnikoff) as described by the Committee as Metchnikoff's race A (p. 36).

CC. Produce balls of amino-acid crystals in meat after a comparatively short period of incubation. Violently proteolytic, may produce an exotoxin, and invade tissue, which is vigorously digested.

Genus 26. *Weinbergillus* nov. gen. Type species *histolyticus* (*Bacillus histolyticus* Weinberg and Séguin) as described by Henry.

#### DEFINITIONS OF CERTAIN GENERA OF THE CLOSTRIDIOIDEAE<sup>3</sup>

GENUS 1. *Clostridium* Prazmowski 1880, emmend. Heller. *Clostridioideae* that do not liquefy gelatin. Most species cause stormy fermentation of milk. Highly saccharolytic, many even splitting pectins. Produce considerable amounts of butyric acid as a split-product of carbohydrate fermentation. May fix nitrogen. Readily derive their nitrogen from inorganic nitrogen salts. Large rods which are frequently polymorphic and form large orgonts and oval spores. Frequently store up starch. Form in deep agar large lenticular or modified lenticular colonies. Common destroyers of plant tissue (not cellulose) in soil. Used in the retting of flax to split pectins.

Type species, *C. amylobacter* van Tieghem as described by Winogradsky (1896). Probable synonyms: *Amylobacter* Trécul, *Clostridium butyricum* Prazmowski, *Amylobakter* Gruber, *Granulobakter saccharo-butyricum* Beijerinck, *B. saccharobutyricus* von Klecki, *Granulobacillus saccharobutyricus mobilis nonliquefaciens* Grassberger and Schattenfroh, *B. amylobacter* von Hibler, *B. amylobacter* Arthur Meyer and Bredemann. Most of these are today incapable of accurate definition and several of them represent groups and not entities. For this reason the type description chosen is that of Winogradsky instead of that of Prazmowski.

<sup>3</sup> For definition see Jour Bact., 6, 550.

Trécul (1865) gave the bacteria that contain starch the name of *Amylobacter*. He (1867) declared the *Amylobacter* to be heterogenetic, formed of minute particles that organize themselves into bacilli in decaying plant tissue. Van Tieghem (1877) named *Bacillus amylobacter* bacilli which contained amorphous starch during their growth stage. He believed such organisms to be the agents of cellulose destruction. Prazmowski (1880) described and figured *Clostridium butyricum*, which though it was in impure culture, was evidently of the type described above. Winogradsky (with Friebes) (1896) first defined a type that can be considered a species; he assigned no name to the organism. He declares that it does not split cellulose, but pectin. It ferments glucose, sucrose, lactose and starch in peptone media.

This type of organism has been described by many workers. It has been most thoroughly discussed by Bredemann. *Bacillus amylobacter* A. M. et Bredemann probably includes all the large starch-storing clostridia described above in the generic definition. In his investigation Bredemann used principally cytological criteria, and essential extensive chemical studies were not made. He regards all differences noted between his strains as fluctuating variations. It would seem illogical from the point of view of the general systematist, to assign merely specific rank to a group of organisms so widespread and of such abundant occurrence as are these soil anaerobes. Bredemann's investigation simply indicates that he did not find means of distinguishing his strains, or perhaps that by his technique he isolated only a restricted group of the general type. Bredemann declares that the power of fixing nitrogen varies in these organisms and cannot be used as a specific character. Bredemann's critique of his cultures is apparently very weak. Thus on page 404 he claims to have changed a Welch bacillus into an amylobacter. He quotes seriously the fantastic conceptions of Grassberger and Schattenfroh with regard to the "denaturing" of anaerobic organisms, and his conception of systematic anaerobic work is that of Lehmann and Neumann. He regards as a variant of *B. amylobacter* what appear to be coccus forms contaminating his cultures.

The chemical activities of this type of anaerobe with regard to end-products are described in some detail by Bredemann and by Grassberger and Schattenfroh. The latter find that amylase is usually present, sucrase very rarely so.

*Clostridium Pastorianum* is the name given to the anaerobic nitrogen-fixing bacillus discovered by Winogradsky (1896 and 1902). This author distinguishes it from other clostridia known to him by the fact that the sporangia only partially disappear from about the spores, forming what he terms a "spore capsule," and by the fact that its fermentative ability is less than is that of most soil clostridia. In peptone media it splits glucose, sucrose, laevulose, inulin, galactose and dextrin, but not lactose, arabinose, starch, rubber, mannitol, dulcitol, glycerol or calcium lactate. Presumably stormy fermentation of milk does not then take place. Obviously the production of stormy fermentation of milk, depending on the splitting of ore sugar, is not to be regarded as a generic character. Grassberger and Schattenfroh found certain strains of their organism which did not attack milk with energy. *Clostridium Pastorianum* is sufficiently well differentiated by Winogradsky from the ordinary *amylobacter* or *butyricum* type to warrant its separation from that type as a separate species. Bredemann regards the "spore capsule" formation described by Winogradsky as a frequent anaerobe character. I have never seen an anaerobe strain which produced the remarkable "spore capsules" figured by Winogradsky. This author isolated *C. Pastorianum* only a few times out of many samples of earth, and he was familiar with the type usually termed *C. butyricum* or *B. amylobacter*.

Gruber distinguished two types of sporulating anaerobic granulose-storing butyric acid bacilli, of which the first is the most like the usual conception of *C. butyricum*.

Beijerinck differentiated his granulobacilli into an anaerobic and an aerobic form: Grassberger and Schattenfroh were unable to confirm this work.

Choukevitch (1911) distinguished three types of amylobacter: one fermented glucose, lactose, starch and hemicellulose; one glucose and lactose only; and a third rarely stored up starch

and had little fermentative power. He (1913) identified as *B. amylobacter* strains which ferment cellulose.

Pringsheim described *C. americanum*, which fixes nitrogen less energetically than *C. Pastorianum* and ferments the same sugars as that organism, besides mannitol, glycerol and lactose. It will grow in open flasks; Bredemann terms it an anaerobe.

Douglas, Fleming and Colebrook have described under the name *B. butyrius* a medium sized bacillus that should be assigned to this genus or to *Multifermentans*. McIntosh describes what may be the same strain.

Prazmowski described under the name *Clostridium polymyxa* an aerobic organism. The generic name has since been used occasionally for aerobes. It must in future be restricted to the group of anaerobic organisms which most strongly resemble the first type described by Prazmowski, *C. butyricum*.

There apparently remains abundant critical chemical and systematic work to be done in the study of this important genus, and such work should be performed with improved technique and with cultures whose purity will stand criticism.

#### GENUS 2. *Omelianskillus* nov. gen.

*Clostridioidae* that do not grow well in media containing much protein, and may derive all their nitrogen from mineral salts. Split cellulose or hemicellulose. Do not contain starch. Long slender bacilli with spherical spores. Colonies may be produced on potato slants; they are minute, yellowish and transparent. Agents of plant putrefaction, found everywhere.

Type species *O. hydrogenicus* (the ferment of cellulose which produces hydrogen, of Omeliansky) as described by Omeliansky (1895 and 1904 b). Characters of genus. Probably several species were studied by Omeliansky. In his later work he admits that the cultures studied by him were not pure. Another type, *O. methanicus*, similar to the above, is said to be the agent of methane formation in cellulose fermentation. This species may be assigned to the same genus.

These organisms were isolated by growing them in a medium free of organic nitrogen. There may be other genera of anaerobic



cellulose splitters which require some organic nitrogen for their metabolism.

Choukevitch (1911) describes under the name *B. gazogenes* an organism of active growth habit and of very strong fermentative powers, that splits starch and hemicellulose. Its morphology is similar to that of Omeliansky's organisms and it may be included in this genus.

Choukevitch (1913) considers his type I of *B. Rodella III* as similar to *O. methanicus* and type II as similar to *O. hydrogenicus*, but his reasons for so doing are not very sound. These two organisms do not split cellulose.

### GENUS 3. *Macintoshillus* nov. gen.

*Clostridioideae* that do not liquefy gelatin. They produce acid and gas and no putrefaction in meat media. They do not readily attack milk and they ferment few or no sugars. Gram-negative rods with parallel sides and with terminal spherical spores. Colonies in deep agar are small and irregular but not woolly. Frequently found in wounds. Apparently incapable of invading tissue.

Type species *tetanomorphus* (pseudotetanus bacillus, McIntosh (p. 32), *Bacillus tetanomorphus* Committee (p. 41)), as described by the Committee. Glucose and maltose are fermented.

*Bacillus tetanoides A* of Adamson (1919) is to be assigned to this group. Acid and no gas is produced from glucose and maltose by the majority of Adamson's strains, while one strain showed no fermentative ability. The former type is probably identical with McIntosh's organism.

Choukevitch (1913) describes as type 1 of the bacillus known as *Rodella III* a slender and highly saccharolytic organism with spherical spores, resembling morphologically Omeliansky's cellulose fermenters. It does not ferment cellulose nor liquefy gelatin and may temporarily be placed here in spite of the fact that it is said to produce hydrogen sulfide. If the organism does not liquefy gelatin it is unlikely that it produces any considerable amount of hydrogen sulfide from protein when in pure culture. This organism is a common intestinal sapro-

phyte of cattle and sheep. A heavier bacillus, type II, is equally difficult to place. It also produces hydrogen sulfide and is highly saccharolytic.

It may be that another genus should be created for highly saccharolytic spherical end-sporing organisms that grow on ordinary media.

GENUS 4. *Douglasillus* nov. gen.

*Clostridioideae* that do not liquefy gelatin. They produce little gas in meat medium. They may clot milk. Gram-negative bacilli which are frequently fusiform and may show peculiar involution forms. Spherical spores are formed in the bacilli; the rods are usually widened by the spores so that their sides are not parallel. Young spores may not be truly spherical. Occasionally found in wounds. Probably incapable of invading tissue.

Type species *sphenoides* (*Bacillus sphenoides* Douglas, Fleming, and Colebrook), as described by the Committee (p. 43): the type which ferments glucose, maltose, galactose, lactose, salicin, mannitol, sucrose, dextrin and starch.

The Committee states that the fermentation reactions of this group are variable. *Bacillus* E of Adamson (1919) is apparently closely related to *D. sphenoides*. It produces peculiar involution forms and though it ferments several sugars (glucose, maltose, lactose and mannitol) it produces very little gas from them, and has not strong fermentative powers.

The pointed rod named *Coccobacillus procaetus* by Tissier may be included in this genus. It does not ferment lactose or sucrose but does attack glucose.

These organisms are not to be confused with the nonsporulating anaerobic fusiform bacilli which show a spotted staining reaction and invade tissue, but grow poorly on artificial media. These latter may be trained to an aerobic habit. They have been placed in the genus *Fusiformis* in the family *Mycobacteriaceae*, order *Actinomyceetales* by the Committee on Characterization and Classification of Bacterial Types, where they may well be left because of the fact that they are highly adapted parasites.

GENUS 5. *Henrillus* nov. gen.

*Clostridioideae* that do not liquefy gelatin. They produce acid and gas in meat medium. They clot milk readily and attack many sugars, producing much acid. Gram-negative or gram-positive slender rods with terminal oval spores. Their colonies in deep agar are large, lenticular and opaque. Very common in soil, often found in wounds, do not invade tissue.

Type species *H. tertius* (*Bacillus tertius* Henry) as described by Henry. Henry believes that the conception *B. tertius* applies to a group and not to a species. His nine strains which may be taken as a type split the monoses, bioses, mannose, xylose, starch, dextrin, glycogen, salicin, amygdalin and mannitol.

This type was described by von Hibler with the number IX. Fleming (Bac. Y), Rodella (1902) (Bac. III), Robertson (1916 a), McIntosh, Adamson (1919) and the Committee and Weinberg and Séguin also describe it. Choukevitch (1913) identifies spherical sporulating organisms with Rodella III. The original Rodella III did not clot milk and should perhaps be associated with *Flemingillus*.

GENUS 6. *Flemingillus* nov. gen.

*Clostridioideae* that do not liquefy gelatin. They produce little gas or acid in meat medium. They do not grow very abundantly in milk or change it. They do not show any marked tendency to split sugars. Slender Gram-negative rods with oval terminal spores. Colonies in deep agar lenticular, may show an areola of fine radiations. Frequently found in wounds, not pathogenic for guinea-pigs.

Type species *F. cochlearius* (*Bacillus cochlearius* Douglas, Fleming and Colebrook) as described by the Committee. This type is highly motile and it split none of the carbohydrates that it was grown in. It was described as bacillus III type C by McIntosh.

Bacillus C of Adamson (1919, p. 380) should be referred to this genus. It is slightly motile. McIntosh's *Bacillus* III A was also considered by Adamson to belong in such a group as this. It splits glucose and maltose only. We may, until the non-

proteolytic slender end-sporing rods have been carefully investigated, include strains that split a few easily fermentable sugars and do not produce much acid in genus *Flemingillus*, and those that split many sugars and tolerate much acid in the genus *Henrillus*.

Choukevitch describes a number of organisms possessing a similar lack of fermentative ability to *Flemingillus*. Possibly his *B. irregularis* belongs here; *Streptobacillus anaerobicus-magnus* does not resemble this type morphologically, but does chemically.

*B. ventriosus* of Tissier and *B. gracilis-putidus* of Tissier and Martelly resemble this type. *Coccobacillus oviformis* and *B. capillosus* of Tissier resemble it in fermentative powers but not in morphology.

#### GENUS 7. *Vallorillus* nov. gen.

*Clostridioideae* that do not liquefy gelatin. They produce gas and acid in meat medium but no digestion. They clot milk slowly and attack various sugars. Rather slender Gram-positive rods with little or no tendency to form spores. Form lenticular colonies, "coeurs jaunes," in deep agar. May invade tissue, producing oedema and gas. Pathogenicity transitory.

Type species *V. fallax* (*Bacillus fallax* Weinberg and Séguin) as described by the Committee (p. 27), the type which ferments glucose, laevulose and maltose.

Henry regards this organism as capable of fermenting many sugars and starch.

Choukevitch (1911) describes under the name *B. bifurcatus gazogenes* a large branching organism which has fermentative reactions similar to *Vallorillus*, but can hardly be included in the genus on account of its unusual morphology.

#### GENUS 8. *Multifermentans* nov. gen.

*Clostridioideae* that do not liquefy gelatin. Produce gas and acid in meat medium. Clot milk readily, without stormy fermentation. Rather small Gram-positive rods with oval central or subterminal spores. Actively saccharolytic. Found occasionally in wounds, not tissue invaders.

Type species *M. tenalbus* (*Bacillus multifermentans-tenalbus* Stoddard) as described by Stoddard (1915 b). This organism, of which Dr. Stoddard was so kind as to send me a culture, does not fit into any other of the genera here defined. Stoddard's organism ferments glycerol, maltose, lactose, raffinose, glucose, sucrose, inulin, and salicin. Mannitol and dulcitol are not fermented.

Adamson describes under the name *B. butyricus* a "small or medium-sized" bacillus that ferments glucose, lactose, maltose and sucrose but not mannitol and starch. A butyric acid odor is produced. Perhaps it should be assigned to this group.

#### GENUS 9. *Hiblerillus* nov. gen.

*Clostridioideae* that do not liquefy gelatin. They do not clot milk. Large gram-positive rods which form more or less reluctantly long ellipsoid spores; they may form orgonts. Colonies in deep agar, small and lenticular or with fine radiations. May be pathogenic for the guinea-pig, producing oedema and gas, or paralysis.

Type species *H. sextus* (*Bacillus VI* of von Hibler), as described by von Hibler (résumé by Weinberg and Séguin, p. 202).

Von Hibler describes another species which he terms VII. These organisms have much in common and we are probably justified in including them in one genus as *Hiblerillus sextus* and *Hiblerillus septimus*. The latter resembles an organism described by Tizzoni and Cattani, according to von Hibler. There are probably many organisms in soil which are pathogenic for rabbits and guinea-pigs when given certain conditions favorable to invasion, which rarely invade under natural conditions or which on account of shy growth habit are missed when they invade in the company of other organisms.

To this genus may be assigned two organisms isolated from the intestine of the horse and described by Choukevitch (1911); they possess similar fermentative ability: the production of acid and no gas in glucose agar. A non-pathogenic one, *Streptobacillus anaerobicus-rectus* may be termed *H. rectus*; another, *H. megalosporus*, produced a fibrino-purulent peritonitis in a guinea-pig.

GENUS 10. *Welchillus* nov. gen.

*Clostridioideae* that liquefy gelatin but do not produce hydrogen sulfid demonstrable by lead-acetate-paper test in blood-broth. They produce much acid and gas on meat medium, but they do not digest it, nor do they digest casein, coagulated serum, or eggwhite. They produce stormy fermentation of milk and attack many sugars vigorously. Their multiplication is extraordinarily rapid; they are killed by their own growth products in acid media, in which they fail to sporulate. Deeply Gram-positive non-flagellate rods with square ends. They produce oval subterminal or median spores in alkaline media, or in media free of fermentable carbohydrate; these spores do not bulge the sides of the bacilli. When growing rapidly the rods are very short, and resemble closely no other type here listed except *Stoddardillus*. When growing slowly the rods are less abundant and longer, and may be mistaken for *Martellillus* and similar organisms. Typically intestinal saprophytes; ubiquitous. Many strains produce toxin and invade tissue, forming gas, and causing the formation of oedema, and in many cases causing the disintegration of muscle and of connective tissue without the production of a foul odor. This disintegration occurs only *in vivo*, and is probably due to the enzymes of the host tissue. *Welchillus* are the most frequent anaerobic invaders noted on the hospital autopsy table: the organisms are present in human intestines. Causative agents of a probably greatly overestimated percentage of gas gangrene cases following war wounds. Comparatively rare as animal invaders.

Type species *W. aerogenes* (*Bacillus aerogenes-capsulatus* Welch and Nuttall) type I as defined by Simonds (type IV of Esty). Ferments, besides other carbohydrates, inulin and glycerol. Usually pathogenic for guinea-pigs.

*Synonyms.* *B. phlegmones-emphysematoseae* Fraenkel, *B. perforans* Veillon and Zuber, 'Bacille de rheumatisme aigu' of Achalme, *Butyribacillus immobilis-liquefaciens* Grassberger and Schattenfroh. Descriptions and discussions, most of them with large bibliographies, are to be found under the names of the following authors: von Hibler, Simonds, Robertson, Wein-

berg and Séguin, Henry, McIntosh, Adamson, The Committee, Jablons, Esty. Simonds divided the group into four sub-groups on the basis of the fermentation of inulin and glycerol. Henry and Esty substantiated this finding. The latter finds his strains divisible into two sub-groups on the basis of the sensitiveness of the spores to heat. The division thus made does not coincide with those secured by means of sugar fermentation. McIntosh admits a species which sporulates on ordinary media. The Committee, on which McIntosh later served, do not mention such a type. The chemical behavior of *Welchillus* has most recently been studied by Wolf (1919) and Wolf and Harris (1917, a, b, and c). Agglutinins are extremely difficult to produce with these organisms, and are found to agglutinate only homologous strains. Werner succeeded in agglutinating one non-homologous strain. Robertson (1916 b) failed to immunize guinea-pigs with bacterial protein. Toxins, according to the work of Bull and Pritchett, are all neutralized by the same antitoxin. Esty immunized guinea-pigs with young whole cultures.

GENUS 11. *Stoddardillus* nov. gen.

*Clostridioideae* of energetic growth habit that liquefy gelatin, but do not produce H<sub>2</sub>S demonstrable by a lead-acetate-paper test in blood broth. Produce abundant gas but little acid in meat medium. Grow very shyly or not at all in milk. Attack a few sugars, but do not produce much acid. Short chunky Gram-positive rods which do not form spores. Colonies in deep agar large, lenticular and opaque. Not easily distinguished from *Welchillus*. May invade tissue, causing considerable destruction of muscle but no foul odor. One strain, reported from a case of human gas gangrene.

Type species *S. egens* (*Bacillus egens* Stoddard) as described by Stoddard (1919 a). Splits glucose, laevulose, mannose, maltose, dextrin, glycogen, inosite and glycerol. Does not sporulate on six days' incubation in inspissated serum.

Such organisms as this are probably not nearly so rare as reports would indicate because they do not sporulate and are

killed in most anaerobic isolation procedures. Because of their close resemblance to *Welchillus* and because of the ubiquity of organisms of that genus their detection is rendered still more difficult.

It may be that later workers will prefer to include this organism in the genus *Welchillus*. The action on milk is dependent on the fermentation of lactose, and sugar fermentations are not to be regarded as of generic significance. I place it in a genus by itself because spore formation has not been demonstrated for this organism.

GENUS 12. *Rivoltillus* nov. gen.

*Clostridioideae* possessing moderately strong saccharolytic powers. Liquefy gelatin but do not produce  $H_2S$  demonstrable by a lead-acetate-paper test in blood broth. Produce in meat medium gas and a pink coloration which does not rapidly fade. Clot milk. Do not liquefy serum or egg or disintegrate meat particles. Gram-positive rods, usually short, with median, sub-terminal, or terminal spores, which usually bulge the sides of the bacillus. Sporangia not often much larger than vegetative rods. In tissue the sporangia may be uneven in their staining reactions, "granulose" being present; orgonts are long, frequently with parallel sides. Usually form chains on the liver of animals. Colonies in deep agar, though they may start as lenticular structures, consist later of a dense center and a wide loose woolly periphery. They vary in size, etc., according to species. Typically highly pathogenic tissue invaders that produce haemolysis and gas in the animal body. Pathogenic for a wide range of species.

Type species *R. vibriion* (the vibriion septique of Pasteur), as defined in a future paper. Robertson (1920) has divided the group into four sub-groups on the basis of the agglutination reaction.

Probably the group of anaerobes whose nature is most frequently discussed. The morphological resemblance of individuals and of colonies to organisms of the sporogenes type led to the frequent description of mixed cultures of the two types,



usually under the name *B. oedematis-maligni*. The literature on this group is too extensive to quote. See Ghon and Sachs, Meyer, Weinberg and Séguin, Robertson, the Committee, Wolf (1918). A review of the animal infections has been made by Heller (1920). Under the name *Bacillus tumefaciens* (not to be confused with the plant pathogen *Bacterium tumefaciens*). Wilson describes an organism similar to those of this genus. His description does not convince the reader as to the purity of the culture studied: a mixture of vibriion septique and oedematis-like organisms would behave as did Wilson's bacillus.

GENUS 13. *Arloingillus* nov. gen.

*Clostridioideae* that attack sugars with considerable energy but have a somewhat restricted action on proteins. Liquefy gelatin but do not produce H<sub>2</sub>S demonstrable by a lead-acetate-paper test in blood broth. In meat medium produce gas and a pink coloration that soon fades. Autoagglutinate readily. Clot milk if blood is present. Do not digest serum or egg. Vegetative forms are small gram-negative rods with even staining; forms about to sporulate are uneven in staining reaction, often far larger than vegetative rods, citron or spindle shaped; orgonts (see Heller), show marked tendency to store up granulose. Spores oval, may vary greatly in length. Bacilli do not form long chains on the liver of animals. Colonies in deep agar lenticular, sometimes showing concentric formation, or compound lenticular. Colonies vary considerably according to species. Typically toxic tissue invaders which produce marked haemolysis. Pathogenic for guinea-pigs, cattle and sheep.

Type species *A. Chauvoei* (*Bacterium Chauvoei* Arloing, Cornevin, and Thomas) as described in a future paper.

This genus contains several species which will be discussed. These organisms show some similarity to those of the genus *Clostridium*.

The *B. enteritidis-sporogenes* Klein, as described by von Hibler (1908) should probably be included in the genus. The only character which is markedly different from that of the genus is the energetic fermentation of milk shown by von Hibler's bacillus IV.

GENUS 14. *Meyerillus* nov. gen.

*Clostridioideae* that produce H<sub>2</sub>S on blood broth and liquefy gelatin. In meat medium they produce gas but little or no pink coloration; they show no marked proteolytic action. Do not readily attack milk. Large Gram-positive rods with little or no inclination to form spores. Attack a few sugars. Colonies in deep agar large, opaque and lenticular. Typically tissue invaders of marked power, attacking and digesting *in vivo* the connective tissue more than the muscle.

Type species *M. sadowa* nov. sp. To be described in a future paper. This organism was at first taken for *B. Welchii*. It does not sporulate. It is one of the four guinea-pig invaders isolated from a case of human gas gangrene. I am inclined to place the sporulating bacillus L of Adamson (1919) in this genus but have not handled that organism. Perhaps it is premature to decide whether sporulation may be used as a generic character. Adamson finds bacillus L very slightly proteolytic on milk and not so on other media. *M. sadowa* does not grow on milk.

GENUS 15. *Novillus* nov. gen.

*Clostridioideae* that in blood broth produce H<sub>2</sub>S demonstrable by lead-acetate-paper test; they liquefy gelatin. Produce gas and on continued incubation produce a pink coloration in meat, but this color rapidly fades. Autoagglutinate with extreme readiness. Slowly attack milk and a few carbohydrates, but do not form much acid. Heavy rods of apparently shy growth habit on most media; form a few oval spores that may or may not bulge the sides of the bacilli. Colonies usually large and opaque, may form slender projections or even long fine woolly filaments. Frequently yellowish. Typically toxic tissue invaders which frequently cause the formation of a thick gelatinous oedema that does not lose its gelatinous consistency on section. May also produce gas and a black haemorrhagic condition of the muscle if rapid invasion by large numbers of bacilli takes place. One strain causes considerable tissue destruction. Pathogenic for guinea-pigs, mice, man, hogs, horses, cattle.

Type species *N. maligni* (*Bacillus oedematis-maligni* II, Novy) as described by Novy.

The lesions typically produced by organisms of this group were first described by Koch. Gohn and Mucha give an elaborate description of an organism which belongs to this genus. Von Hibler studied four strains of "Novy's bacillus." Descriptions of organisms of this type have been given by Kerry, by Rivas and by Diedrichs. Weinberg and Séguin described *B. oedematiens* which is different from *N. maligni* but is to be included in this genus. *B. bellouensis* Saquépée also belongs in this genus, as may also the organism called by Adamson bacillus S (1919, p. 373). I have found a type that was rapidly fatal to horses, and another different somewhat from all the rest in a septic wound, and hope to define these species accurately in another paper. Wolf (1920) has studied the biochemistry of an organism of this group.

#### DEFINITIONS OF CERTAIN GENERA OF THE PUTRIFICOIDEAE<sup>4</sup>

##### GENUS 16. *Seguinillus* nov. gen.

*Putrificoideae* that do not exert a marked action on protein. Soften and may blacken meat. Milk clotted, may later be digested. Attack a few sugars. Very reluctant to form spores. Minute Gram-positive or Gram-negative rods, uniform in thickness but not in length. Spores oval, sub-terminal. Deep colonies lenticular or modified lenticular. Occasional tissue invaders which produce a proteolytic gangrene or phlegmon.

Type species *S. aerofoetidus* (*Bacillus aerofoetidus* Weinberg and Séguin) as described by Weinberg and Séguin (p. 161). The organism forms oval subterminal spores.

These may well be called "borderline" organisms. Henry places them in his "saccharolytic group" on account of their sugar-splitting proclivities. A strain given by me by Weinberg does not grossly attack meat particles.

##### GENUS 17. *Regillus* nov. gen.

*Putrificoideae* that show to the eye little proteolytic action on meat. They may or may not blacken it slightly, but the

<sup>4</sup> For definition see Jour. Bact., 6, 550.

meat particles do not diminish in size. Sugars are attacked. Milk is digested. Gram-positive rods which readily form oval subterminal or median spores. Colonies minute, opaque, complex, yellow. Typically toxic tissue invaders which produce in the guinea-pig a clear white oedema that does not rapidly lose its oedematous nature on section.

Type species *R. progrediens* nov. sp. To be described in a future paper. Two species, one found in a case of human gas gangrene (see Barney and Heller), the other in a sheep.

GENUS 18. *Robertsonillus* nov. gen.

*Putrificoideae* that produce on meat medium a little gas and a terra cotta coloration, multiplying slowly for a long period. A black pigment is absent. Sugars not fermented. Weakly Gram-positive or Gram-negative rods with oval spores, usually subterminal, that somewhat distend the bacillus. Two spores often occur in one rod. In old cultures enormous snakey Gram-negative rods are produced. One species, found twice in wounds. Non-pathogenic for guinea-pigs in pure culture.

Type species *R. primus* (Bacillus I, Hempl) as described by Hempl. Descriptions of bacilli similar to this have not been noted. Proteolytic organisms producing a terra cotta coloration in meat medium are not uncommon, however.

GENUS 19. *Nicolaierillus* nov. gen.

*Putrificeoidae* that in meat medium produce gas and various color changes: yellowish, pink, grey or mauve, depending on the medium; the particles of meat are gradually suffused with a black pigment, and bleach at the top. The meat is softened but the particles do not greatly diminish in size. Do not attack sugars. Gram-negative (weak methyl violet) rods that form terminal spherical spores. Colonies in deep agar diverse. Common in soil, found in horse feces, may multiply in wounds, but do not normally invade tissue. Produce a characteristic neuro-toxin.

Type species *N. tctani* (*Bacillus tctani* Nicolaier), to be described in a future paper.

Tulloch (1917 and 1919) has divided the group into four types on the basis of the agglutination reaction. Adamson (1920)

describes the cultural behavior of five strains of *B. tetani*. Two papers on the behavior of my cultures are forthcoming.

This is not the genus *Plectridium* of Fischer. *Plectridium* included some butyric acid bacteria, the tetanus bacillus, a putrefactive organism, *Plectridium putrificum*, and other genera not yet known; and it embraces, according to my scheme of classification, elements altogether incoherent. Were the descriptions fuller or were I better acquainted with the proteolytic group, two more genera of proteolytic spherical end-sporers might be suggested: Those that blacken meat readily, e.g., *B. cadaveris-sporogenes*, Klein; and those which do not blacken it at all, e.g., *B. tetanoides* B of Adamson.

GENUS 20. *Martellillus* nov. gen.

Hardy *Putrificoideae* that in meat medium multiply rapidly at an early stage of incubation, producing a greyish coloration and later a blackish deposit on the meat particles, and after three days' incubation cease to multiply actively. Sporulate early in the development of a culture, later cease to do so but vegetate very slowly. Produce very little gas in meat medium. Digest milk. Attack a few sugars. Heavy deeply gram-positive rods, may vary greatly in size. Spores usually cocoon-shaped, usually median or sub-terminal, do not greatly bulge the sides of the bacillus. Colonies in deep agar lenticular, irregular, or stellate. Common putrefactive organisms that readily invade tissue in company with other organisms, producing a greenish proteolytic gangrene. I have found them in the heart's blood and organs of a woman dying of pernicious vomiting and uraemic poisoning and apparently the only invader in a mouse dying from an otherwise unknown cause. The Committee find *B. bifermentans* in acute cases of gas gangrene. Weinberg and Séguin state that such an organism invades guinea-pigs in company with *B. perfringens*. It is possible that *Martellillus* bacilli produce metabolites poisonous to themselves and to animals and that cause early sporulation. They frequently cease active multiplication when the culture has a reaction near pH 7.0.

Type species *M. bifermentans* (*Bacillus bifermentans-sporogenes* Tissier and Martelly) as defined by Tissier and Martelly.

Hempl has described a similar organism not identical with that of Tissier and Martelly (Organism II, which may be renamed *Martellillus proteolyticus*). Bacillus II of Choukevitch probably belongs in this genus, as does von Hibler's bacillus XV. *B. sporogenes* B, as described by Choukevitch also belongs here. A rather shyly growing species sent me by Major Nichols which was named *B. bellonensis* Saquépée, was apparently pure, non-pathogenic, and referable to this genus. It was not the organism described by Saquepee as *B. bellonensis*, nor did it resemble other strains sent me by Saquépée. Adamson describes two species of this type. One under the name of *B. bifermensporogenes*, which is non-motile and does not split sugars, the other under the name of "Central spore bacillus" which he identifies as McIntosh's type XII, which is motile and splits glucose and maltose. The type species is, however, supposed to be saccharolytic. McIntosh's type XII (*parasporogenes*) does not behave in my hands as does this latter type of Adamson's and I should not include them in the same genus. McIntosh's type XIII, a pathogenic proteolytic organism, should probably be placed here. Some of the organisms here listed are probably identical, but one is not warranted in so considering them without a direct comparison of strains. Study of many strains would probably necessitate the division of this genus into two, on the basis of carbohydrate fermentation.

This is one of the important groups that most need systematic investigation.

GENUS 21. *Recordillus* nov. gen.

*Putrificoideae* that, though they sporulate, are exceedingly delicate and soon die in meat medium and other media. Their growth in meat medium resembles that of the organisms of genus *Martellillus*; they do not produce much gas, they color the meat particles a greyish color and form a blackish pigment. Gram-negative rods with central or sub-terminal cocoon-shaped spores. Parasitic forms which infect cattle in California and Nevada. I venture to place such organisms in a separate genus on account of their parasitic habit and on account of their

delicacy. It may be that they are descended from organisms of the genus *Martellillus*.

Type species *R. fragilis* nov. sp. Characters of genus.

I regret that the strain kindly sent me by Dr. Records died soon after its arrival. As it had sporulated in meat medium this was surprising, but was consistent with Dr. Records' description of the behavior of the organism. The strain was isolated from a liver infaret in a cow.

GENUS 22. *Tissicrillus* nov. gen.

*Putrificoideae* that attack sugars and clot milk. Slender Gram-negative or Gram-positive rods with oval terminal spores. Colonies in deep agar have radiate periphery and opaque center. Frequently intestinal saprophytes.

Type species *T. paraputrificus* (*Bacillus paraputrificus* defined by Bienstock) as described by McIntosh under the name *Bacillus putrificus*. Ferments glucose, maltose, lactose, sucrose and starch.

References to organisms of this type are made by Passini, Moro, Metchnikoff and Kligler. Very likely some descriptions of this type were based on the behavior of mixed cultures.

GENUS 23. *Putrificus* nov. gen.

*Putrificoideae* that do not attack sugars. Slender Gram-negative or Gram-positive rods with oval terminal spores. Colonies in deep agar have radiate periphery and opaque center. Putrefactive organisms found in soil and wounds.

Type species *P. Bienstocki* (*Bacillus putrificus* Bienstock) as defined by Bienstock (1906). References: Bienstock (1884, 1901, 1906), Klein, Rodella (1905). The latter differentiated three types of what he termed *B. putrificus*. Tizzoni, Catani and Baquis described two organisms of this general type. *B. postumus* of Würcker is of this group. His *B. putrificus* is of sporogenes affinities. I possess a strain (IOR) isolated by Miss Robertson or myself from a wound, which is mildly proteolytic, does not split sugars, is Gram-negative, and on serum produces oval terminal spores. Its deep colonies are transparent, len-

ticular, with a protruding fluff. Dr. Meyer tells me that he occasionally encounters such organisms, very slow in initial activity in pure culture, but more active in mixed culture, mildly proteolytic in their action on meat. The colony is not unlike that of *B. botulinus*.

The Committee find that many so-called "*Putrificus*" strains consist of a non-proteolytic oval end-spore contaminated with a sporogenes-like organism. In the recent German literature "*Putrificus*" refers usually to the sporogenes type. Metchnikoff's strains termed *Putrificus* do not resemble closely those described by Bienstock.

GENUS 24. *Ermengemillus* nov. gen.

*Putrificoideae* that produce a yellowish coloration in meat medium, and later blacken and digest it. They are more highly proteolytic than the organisms of the foregoing genera. They ferment various sugars. Gram-positive rods which form sub-terminal oval spores. Their colonies in deep agar are, when discrete, lenticular or kidney shaped, and may show tufted smooth or woolly polar projections and infrequently fine loose woolly radiations. Fairly common in soil, grow readily in vegetable material and on meat. Form a characteristic and powerful neuro-toxin.

Type species *E. botulinus* (*Bacillus botulinus* van Ermengem) as described by K. F. Meyer and co-workers in a future paper.

Leuchs discovered that there were two types of toxin produced by different European strains. Burke found two types of toxin produced by different American strains. Probably a number of species may be distinguished by careful methods.

McIntosh and the Committee find that *B. botulinus* shows slight proteolytic power, and meat is stated to be a poor medium for the growth of the organism. My experience has been very different from theirs. Indubitably pure cultures of *Ermengemillus* grow readily on meat medium (pH 7.2) and may show obvious putrefaction in a few days when incubated anaerobically at 37°. But the meat particles are not rapidly diminished in size by the proteolytic action. The observers who did not note the



proteolytic action of these organisms on meat must have used an acid meat medium, or a substratum poor in peptones and peptids, or must have incubated their cultures at room temperature. Von Hibler found that the organism blackened brain medium.

GENUS 25. *Metchnikovillus* nov. gen.

Highly proteolytic *Putrificoideae* that readily blacken meat. They do not produce in it abundant amino-acid crystals, but digest meat, serum, egg and casein rapidly, forming more alkali or less fatty acid than do the organisms of the succeeding group. They split few sugars. Gram-positive or weakly Gram-positive rods, vegetative forms uniform and considerably smaller than sporangia. Sporulate readily in ordinary media, forming oval spores which are usually sub-terminal, though in some strains median spores predominate. Multiplication is exceedingly active, forty-eight hour colonies in deep agar are large and woolly. Frequently a few colonies are larger than the others but they do not give rise in a following generation to a preponderating number of large colonies. Ubiquitous. Common intestinal organisms, abundant in soil; very common in infected wounds. Not capable of invading in pure culture in moderate doses, may invade in company with other organisms or alone when given in large doses.

Type species *M. sporogenes* (*Bacillus sporogenes* type A of Metchnikoff) as described by the Committee (p. 36). Klein described as *B. enteritidis-sporogenes* a mixed culture which contained a non-proteolytic organism, a pure culture of which was described by von Hibler as *B. enteritidis-sporogenes* Klein (von Hibler IV). This tissue invading pathogen, thought by some to have been *B. Welchii*, is most nearly referable to the genus *Arloingillus*. The strain was derived from patients with enteritis and it was apparently contaminated with a proteolytic organism of the genus *Metchnikovillus*. Metchnikoff described two types, A and B, of intestinal anaerobes which he thought were similar to the organism of Klein, and which he termed *B. sporogenes*. His descriptions permit of no identifications. Choukevitch, working in Metchnikoff's laboratory described *B. sporogenes* A and B more carefully. Type B should be referred to genus

*Martellillus*. Weinberg made the identification of type A more exact, and the Committee discuss it at some length. They allow two species, *B. sporogenes* type A of Metchnikoff, and *B. parasporogenes* McIntosh, which are different in colony formation and serologically. Donaldson describes the "Reading" bacillus, to be assigned to this genus. Superficial acquaintance with many strains of proteolytic anaerobes leads me to suggest that *Metchnikovillus* may be defined as a genus of many species; Dr. K. F. Meyer is also of this opinion. Henry (p. 361) believes it likely that the conception "sporogenes" refers to a group of organisms.

Barger and Dale, Weinberg and Séguin, M. E. Bullock and the Committee discuss the poisonous growth product of this type of organism: it is apparently a lower split-product than are true toxins. Some of these authors find that the culture filtrate of *B. sporogenes* increases the toxicity and invasive power of *B. Welchii*. Donaldson and Joyce placed the Reading bacillus in wounds to digest necrosed muscle and report no accidents due to its presence. Wolf (1919), Wolf and Telfer, and Wolf and Harris (1917, a and b) describe the chemical activities of these and related organisms.

Because of their universal occurrence and active growth habits, organisms of this type frequently contaminate anaerobic cultures. Their chemical activities are thus described in conjunction with the pathogenic properties of the cultures in which they are active. The descriptions which tally more or less accurately with the sporogenes type are legion. Thus *B. oedematis maligni* Koch of von Hibler was probably a mixture of an organism of this genus with a chain-forming vibriion septique; while bacillus XI of von Hibler (1908) was probably such a mixture with a similar pathogen which formed chains somewhat reluctantly (Pl. II, fig. 3). I am today able to discover only the sporogenes type in von Hibler's strain of bacillus XI. Whether bacillus XIX of McIntosh should be included in the genus *Metchnikovillus* is difficult to state. It is apparently an active tissue invader and forms smooth lenticular colonies.

When surface methods of isolation are followed this type of organism is very difficult to remove from cultures of other anaerobes. The Committee emphasize this point. I have had no more contaminations by sporogenes than by aerobes and by various other anaerobes. The organism is common in nature and gains entrance to pure cultures occasionally.

GENUS 26. *Weinbergillus* nov. gen.

Highly proteolytic *Putrificoideae* that in meat medium cause the formation of balls of amino-acid crystals. They digest the meat particles till their bulk is greatly reduced and form large amounts of ammonia, amino-nitrogen and fatty acids. They digest the casein in milk rapidly. Attack few or no sugars. Produce little or no gas in agar media. Gram-positive or Gram-negative rods with sub-terminal oval spores. Colonies in deep agar small, delicate woolly structures. May invade living tissue in company with other organisms, or at times alone, producing a rapid and complete digestion of muscular and connective tissue structures.

Type species *W. histolyticus* (*Bacillus histolyticus* Weinberg and Séguin) as described by Henry (p. 370). Weinberg and Séguin and the Committee allow considerable variation in characters for the strains termed by them *Bacillus histolyticus*.

It is possible that *B. sporogenes-parvus* of Choukevitch may belong in this genus. Wolf and Harris (1918) have made a chemical study of what is probably the type strain—they received it from Henry who received it from Weinberg.

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INDEX TO NAMES OF BACTERIA

- Actinomyces talus*, 2; 14  
*Amylobacter*, 9 ff.  
*Arloingillus*, 6; 21; 29  
 ——— *Chauvoci*, 6; 21  
 Bacille de rheumatisme aigue, 18  
*Bacillus*, 1  
 ——— *acrofoetidus*, 4; 7; 23  
 ——— *acrogenes capsulatus*, 6; 18  
 ——— *amylobacter*, 9 ff.  
 ——— *bellonensis*, 23; 26  
 ——— *bifermentans sporogenes*, 4; 8; 25  
 ——— *bifurcatus gazogenus*, 16  
 ——— *botulinus*, 4; 8; 28  
 ——— *butyricus (Clostridium)*, 5; 12; 17  
 ——— of Adamson, 17  
 ——— *cadaveris sporogenes*, 25  
 ——— *capillosus*, 16  
 ——— *cochlearius*, 5; 15  
 Bacillus C of Adamson, 15  
*Bacillus cogens*, 6; 19  
 ——— *enteritidis sporogenes*, 21; 29  
 Bacillus E of Adamson, 14  
*Bacillus fallax*, 6; 16  
 ——— *gazogenes*, 13 ff.  
 ——— *gracilis putidus*, 16  
 ——— *histolyticus*, 4; 9; 31  
 ——— *hydrogenicus*, 12  
 ——— *irregularis*, 16  
 Bacillus L of Adamson, 22  
*Bacillus megalosporus*, 17  
 ——— *methanicus*, 12  
 ——— *multifermentans tenalbus*, 6; 16  
 ——— *oedematis*, 4; 23  
 ——— *oedematis maligni*, 21; 30  
 ——— ——— II, 7; 23  
 ——— *paraputrificus*, 8; 27  
 ——— *parasporogenes*, 26; 30  
 ——— *perfringens*, 18; 25  
 ——— *phlegmones emphysematosae*, 18  
*Bacillus postumus*, 27  
 ——— *putrificus*, 8; 27; 28  
 Bacillus Rodella III, 13; 15  
*Bacillus saccharobutyricus*, 9  
 Bacillus S of Adamson, 23  
*Bacillus sphenoides*, 5; 14  
 ——— *sporogenes*, 9; 29 ff.  
 ——— ——— B, 26; 29 ff.  
 ——— ——— *parvus*, 31  
 ——— *tertius*, 5; 15  
 ——— *tetani*, 7; 24; 25  
 ——— *tetanooides*, A 13  
 ——— ——— B, 25  
 ——— *tetanomorphus*, 5; 13  
 ——— *tumefaciens*, 21  
 ——— *ventriosus*, 16  
 ——— *Witchii*, 4; 22; 30  
 Bacillus Y of Fleming, 15  
 ——— II of Choukevitch, 26  
 ——— I of Hempl, 7; 24  
 ——— II of Hempl, 26  
 ——— III A of McIntosh, 15  
 ——— III C of McIntosh, 15  
 ——— XII of McIntosh, 26  
 ——— XIII of McIntosh, 26  
 ——— XIX of McIntosh, 30  
 ——— III of Rodella, 13; 15  
 ——— IV of von Hibler, 29  
 ——— VI of von Hibler, 6; 17  
 ——— VII of von Hibler, 17  
 ——— IX of von Hibler, 15  
 ——— XI of von Hibler, 30  
*Bacterium Chauvoci*, 6; 21  
 ——— *tumefaciens*, 21  
 Bifermentans, 4  
*Butyribacillus immobilis liquefaciens*, 18  
 Central spore bacillus, 26  
*Clostridiaceae*, 1; 3  
*Clostridioideae*, 1; 9 ff.  
*Clostridium*, 5; 9 ff. 21  
 ——— *americanum*, 12  
 ——— *amylobacter*, 9 ff.  
 ——— *butyricum*, 5; 9 ff.

- Clostridium, Pastorianum*, 3; 11  
 ——— *polymyxa*, 12  
*Coccobacillus oviformis*, 16  
 ——— *procacutus*, 14  
*Douglasillus*, 5; 14  
 ——— *procacutus*, 14  
 ——— *sphnoides*, 5; 14  
*Ermengemillus*, 8; 28  
 ——— *botulinus*, 8; 28  
*Eubacteriales*, 2  
*Flemingillus*, 5; 15  
 ——— *cochlearius*, 5; 15  
*Fusiformis*, 14  
*Granulobacillus saccharobutyricus mobilis nonliquefaciens*, 9  
*Granulobacter saccharobutyricum*, 9  
*Henrillus*, 5; 15; 16  
 ——— *tertius*, 5; 15  
*Hiblerillus*, 6; 17  
 ——— *megalosporus*, 17  
 ——— *rectus*, 17  
 ——— *septimus*, 17  
 ——— *sextus*, 6; 17  
*Macintoshillus* 5; 13  
 ——— *tetanomorphus*, 5; 13  
*Martellillus*, 8; 18; 25; 26; 30  
 ——— *bifermentans*, 8; 25  
 ——— *proteolyticus*, 26  
*Metchnikovillus*, 9; 29 ff.  
 ——— *sporogenes*, 9; 29  
*Meyerillus*, 7; 22  
 ——— *sadava*, 7; 22  
*Multifermentans*, 6; 12; 16  
 ——— *tenalbus*, 6; 17  
*Mycobacteriaceae*, 14  
*Nicolvierillus*, 7; 24  
 ——— *tetani*, 7; 24  
*Novillus*, 7; 22  
*Novillus, bellonensis*, 23  
 ——— *maligui*, 7; 22  
 ——— *oedematiens*, 23  
*Omelianskillus*, 5; 12  
 ——— *gazogenes*, 13  
 ——— *hydrogenicus*, 5; 12  
 ——— *methanicus*, 12  
*Plectridium*, 25  
 ——— *putrificum*, 25  
*pseudotetanus bacillus*, 5; 13  
*Putrificoides*, 1; 23 ff.  
*Putrificus*, 27; 28  
 ——— *Bienstocki*, 8; 27  
*Reading bacillus*, 30  
*Recordillus*, 8; 26  
 ——— *fragilis*, 8; 27  
*Reglillus*, 4; 7; 23  
 ——— *progrediens*, 7; 24  
*Rivollillus*, 6; 20  
 ——— *vibrion*, 6; 20  
*Robertsonillus*, 7; 24  
 ——— *primus*, 7; 24  
*Seguinillus*, 7; 23  
 ——— *acrofoetidus*, 7; 23  
*Stoddardillus*, 6; 18; 19  
 ——— *egens*, 6; 19  
*Streptobacillus anaerobicus magnus*, 16  
 ——— *rectus*, 17  
*Tissierillus*, 8; 27  
 ——— *paraputrificus*, 8; 27  
*Vallorillus*, 6; 16  
 ——— *fallax*, 6; 16  
*vibrion septique*, 1; 6; 20  
*Wcinbergillus*, 9; 31  
 ——— *histolyticus*, 9; 31  
*Welch bacillus*, see *Welchillus*  
*Welchillus*, 6; 18; 19; 20; 22  
 ——— *acrogenes*, 6; 18

# STUDIES UPON AGGLUTINATION IN THE COLON-TYPHOID GROUP OF BACILLI

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## THE PREVENTIVE ACTION OF FORMALIN IN THE AGGLUTINATION TEST

Malvoz in 1897 claimed that formalin produced chemical agglutination of *Bact. typhosum*, but not of *Bact. paratyphosum* and *Bact. coli*, while, on the other hand Becc, Remy, Widal and Nobecourt were unable to confirm Malvoz's observations.

By adding amounts of formalin from 0.05 to 5 per cent to broth or agar cultures, I have obtained no evidences of chemical agglutination with *Bact. paratyphosum* A and B, *Bact. dysenteriae* and *Bact. coli*; with concentrations of 0.05 to 0.3 per cent formalin spontaneous agglutination in these cultures was almost or entirely prevented while *Bact. typhosum* and *Bact. paratyphosum* A showed spontaneous agglutination without formalin but none with it, when the above dilutions were used. When concentrations of 1 to 5 per cent were used slight spontaneous agglutination sometimes occurred. Spontaneous agglutination was more or less inhibited in all dilutions with *Bact. paratyphosum* B. With *Bact. coli* there was absolutely no change in spontaneous agglutination when formalin was added.

From the above facts it is evident that a concentration of 0.05 to 0.2 per cent formalin gives the most satisfactory results in the prevention of spontaneous agglutination, correct reading being possible at the end of two hours. However, it is a better rule to let the tubes stand over night.

Formalin prevents spontaneous agglutination, but does not interfere with agglutination by specific immune sera; it does, in fact, increase the agglutinating reaction, when used with specific serum.

It has been customary to add formalin as an antiseptic, or to prohibit the growth at certain times of the cultures of the colony-typhoid group, which were to be used in conducting the agglutination test. This method was adapted by Loele, who used 2 per cent formalin, while Porges, Coles, Bass and Watkins,

TABLE 1

*Standardization of formalin concentration in plain broth culture media to prevent spontaneous agglutination. (Cultures twenty-four hours at 37°C.)*

ORGANISM.....	BACT. TYPHOSUM				BACT. PARATYPHOSUM A				BACT. PARATYPHOSUM B				BACT. COLI			
	No. 1	No. 2	No. 3	No. 4	No. 1	No. 2	No. 4	No. 6	No. 1	No. 2	No. 3	No. 5	No. 1	No. 3	No. 5	No. 8
Number of strain.....																
Spontaneous agglutination on cultures.....	4	3	3	3	2	2	3	2	3	3	3	3	3	3	3	4
Dilutions of formalin:																
0.05 per cent.....	0	0	0	0	0	0	0	0	1	1	1	2	3	4	3	3
0.1 per cent.....	0	0	0	0	0	0	0	0	1	1	1	2	3	4	3	3
0.3 per cent.....	0	0	1	0	0	0	1	0	1	1	1	2	3	4	3	3
1.0 per cent.....	0	0	1	0	1	0	1	0	2	1	1	2	3	4	3	3
5.0 per cent.....	1	0	1	0	2	1	1	1	2	1	2	2	3	4	3	3
Control no formalin...	4	2	3	3	2	2	2	2	3	2	2	3	3	4	3	3

*Key:* 5, complete; 4, almost complete; 3, weak; 2, very weak; 1, trace; 0, negative agglutination.

Buxton and Vaughan, Rolly, and Asser used 1 per cent, Widal 0.67 per cent, Garrow, Chick 0.1 per cent; Neisser, Proscher, Lion, Martineck, Ehram, Flatau and Wilke, and Selter also used formalin and showed, that it had a tendency to slow down or diminish the reaction.

Dreyer has made an especially careful study of the use of formalin. He found that 0.1 per cent formalinized broth cultures agglutinate more actively than those without formalin, and that 0.5 to 1 per cent formalin cultures agglutinate less actively than those without formalin.

In my experiments (table 2) using *Bact. typhosum* and *Bact. paratyphosum* A and specific immune serum, agglutination was strongest with tests containing 0.05 to 0.1 per cent formalin; 0.3 per cent showed slightly less agglutination and 1 per cent

TABLE 2

Standardization of formalin concentration in plain broth culture media to promote specific serum agglutination. (Cultures of twenty-four hours at 37°C.)

ORGANISM .....	BACT. TYPHOSUM						BACT. PARATYPHOSUM A						BACT. PARATYPHOSUM B					
	No. 1	No. 2	No. 3	No. 1, 2	No. 2, 3	Total 5	No. 2	No. 3	No. 6	No. 1, 2	No. 4	Total 5	No. 1	No. 3	No. 5	No. 1, 2	No. 5, 2	Total 5
Number of strains																		
Spontaneous agglutination on cultures.....	0	0	0	4	3		0	0	0	2	2		0	0	0	3	3	
Dilutions of formalin and specific serum:																		
0.05 per cent.	4	5	5	5	5	24	5	5	5	5	5	25	5	5	5	5	4	24
0.1 per cent.	4	5	5	5	5	24	5	5	5	5	5	25	5	5	5	5	4	24
0.3 per cent.	4	4	5	4	5	22	5	4	4	5	5	23	5	4	4	5	4	22
1.0 per cent.	3	4	4	4	4	19	4	4	3	4	4	19	4	4	4	4	4	20
5.0 per cent.	2	3	3	4	3	15	3	3	3	3	4	16	3	3	3	4	4	17
Control, 0.....	3	4	4	4	4	19	4	4	4	4	4	20	5	4	4	4	4	21
Control with-out serum:																		
0.05 per cent.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	4
0.1 per cent.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	4
0.3 per cent.	0	0	0	1	0	1	0	0	0	1	0	1	0	0	0	1	3	4
1.0 per cent.	0	0	0	1	0	1	0	0	0	1	1	2	0	0	0	1	3	4
5.0 per cent.	0	0	0	1	1	2	0	0	0	2	1	3	0	0	0	2	3	5
Control, 0.....	0	0	0	4	3	7	0	0	0	2	1	3	0	0	0	3	3	6

Key: 5, complete; 4, almost complete; 3, weak; 2, very weak; 1, trace; 0, negative agglutination.

produced about the same results as those obtained without formalin. With *Bact. paratyphosum* B, agglutination by specific serum in the presence of 0.05 to 0.3 per cent formalin was about the same as, or slightly stronger than, without formalin; with

1 to 5 per cent formalin the agglutination reaction was less than without formalin. However, it has been difficult to standardize this test with formalin, because the reaction depends on the strain of microorganism employed. Many bacilli showed agglutination in all dilutions used and only a few showed different degrees of agglutination; I found that 0.05 to 0.2 per cent formalinized serum gave the best results for most of the strains employed.

#### TECHNIQUE

Since in plain broth or agar cultures spontaneous agglutination interferes with specific agglutination, in such cultures accurate tests are impossible. When spontaneous and non-spontaneous agglutination types are mixed together, I have observed two types of colonies, (by streak on agar plate) occurring regularly throughout the cultures. One type of colony has a smooth surface and regular outline and shows non-spontaneous agglutination, while the other type of colony has a rough surface and irregular outline and is spontaneously agglutinating.

Either type of organism would, however, produce both types of colonies after growing for some time. Some strains changed in a few days, while others remained true to type, producing characteristic colonies even after continual transplanting for over a year. Cultures used in the experimental work were obtained before each set of experiments by plating and fishing a characteristic colony into experimental media.

Only neutral broth cultures gave uniform results. Therefore, 18 to 24 hour cultures in this medium at 37°C., could be used as an indicator of spontaneous and non-spontaneous agglutinations.

The method of my investigations has been to mix 0.3 of each suspension of bacterial cultures in each medium, (such as broth, pepton water and so forth) with 0.3 cc. of the serum dilution, with formalin or without, or with other experimental fluids in small test tubes, of the usual Wassermann type. The tubes, were left at room temperature (excepting in the experiments on temperature), and the preparations were examined, both

macroscopically (by means of a hand lens) and microscopically. In the latter case, as many as 15 or more mixtures were examined at the same time, by transferring a small loopful of each to a square on a large thin glass slide, which had been marked off in squares by means of a grease pencil.

USE OF FORMALINIZED SPECIFIC SERUM IN AGGLUTINATION TEST  
WITH VARIOUS BACILLI (TABLE 3)

I have observed that specific serum to which has been added 0.2 per cent formalin (final 0.1 per cent) in 0.85 per cent salt solution shows stronger agglutination than occurs without formalin. The formalinized serum mixed with an equal amount of broth culture after three hours at room temperature, shows strong agglutination with *Bact. typhosum* and *Bact. paratyphosum* A, and the same is true, if tubes are allowed to stand over night. *Bact. paratyphosum* B and the *Bact. dysenteriae* group show a weak reaction, when examined within three hours; but a stronger reaction if kept over night. *Bact. coli* shows weak formalin readings after three hours; but if kept over night, the result, with or without formalin, is the same.

For the graphical agglutination test, we must use non-spontaneous agglutination bacilli. However, by the addition of formalin, spontaneous agglutination is prevented while at the same time a specific agglutination regularly occurs. As shown in table 3 of the strains of spontaneous agglutination bacilli, *Bact. typhosum* and *Bact. paratyphosum* A yielded specially good results; *Bact. paratyphosum* B showed less influence, while *Bact. coli* was not influenced at all. When the tests were conducted without formalin, spontaneous and specific serum agglutinations usually present the same appearance microscopically and macroscopically, although certain strains of spontaneous agglutination bacilli sometimes yield weaker macroscopic reactions. It is evident that spontaneously agglutinating bacilli should not be used for graphical agglutination tests, and that formalin may be used to prevent the complex reaction.





ACTION OF FORMALIN ON PSEUDO AGGLUTINATION IN THE CROSS  
AGGLUTINATION REACTION WITH DIFFERENT  
IMMUNE SERA (TABLE 4)

In conducting cross agglutination tests with various immune sera pseudo agglutination usually did not take place with formalin. However, certain strains of bacilli showed marked agglutination, as well as specific agglutination, without formalin.

*Bact. typhosum*. Non-spontaneous agglutinating bacilli tested with the formalin showed very marked prevention of pseudo agglutination in these immune sera: *Bact. paratyphosum* A, and B, in horse serum, and even better in *Bact. dysenteriae* Shiga serum, but showed strong spontaneous agglutination without it.

The serum of polyvalent dysentery (1:2000), with *Bact. typhosum* had almost the same agglutinating reaction as the *Bact. dysenteriae* group, both with or without formalin. Hence, we may use the serum for identification of *Bact. typhosum* as well as for the *Bact. dysenteriae* group.

*Bact. paratyphosum* A. Formalinized *Bact. typhosum* serum (1:200), prevented pseudo agglutination entirely, with non-spontaneous agglutinating strains, while showing strong pseudo agglutination without formalin.

In the *Bact. paratyphosum* B serum (1:50), with non-spontaneous agglutinating bacilli, both with or without formalin, agglutination does not appear. This serum showed no tendency to cause pseudo agglutination for *Bact. paratyphosum* A.

Polyvalent dysentery serum, has agglutinated *Bact. paratyphosum* A, in almost the same degree with and without formalin; this serum seems to have a tendency to agglutinate this bacillus, but in this experiment the serum was highly concentrated 1:100. This reaction does not compare with the agglutinating reaction of *Bact. typhosum* or the *Bact. dysenteriae* group, when a dilution of 1:2000 or more is used.

*Bact. dysenteriae* Shiga serum (1:50), showed negative agglutination, both with and without formalin; this serum has no tendency to agglutinate with *Bact. paratyphosum* A.

Normal horse serum (1:50), shows very weak agglutination when using formalin, and stronger without it, but this serum shows a tendency to agglutinate only with *Bact. paratyphosum* A.

TABLE 4

Comparative study of pseudo agglutination with various sera using 20-hour cultures at 37°C., in 0.1 per cent formalized salt solution (0.85 per cent) or normal salt solution (0.85 per cent) with neutral broth in diuum.

IMMUNE SERA FROM RABBIT OR SHEEP; TITRES OF SERUM	ORGANISM.....	BACT. TYPHOSI 3			BACT. PARA A			BACT. PARA B			BACT. COLI			BACT. DYSENTERIAE				
		No. 1	No. 2	No. 3	No. 1	No. 2	Total 3	No. 1	No. 2	Total 3	No. 1	No. 2	No. 3	Total 3	Flex	Shiga	Total	
	Number of strains.....	No. 1	No. 2	No. 3	No. 1	No. 2	Total 3	No. 1	No. 2	Total 3	No. 1	No. 2	No. 3	Total 3	Flex <td>Shiga <td>Total</td> </td>	Shiga <td>Total</td>	Total	
1:30,000	Formalin Salt solution	0	0	0	3	0	3	0	0	0	0	0	0	0	0	0	0	0
1:500	Formalin Salt solution	4	4	5	13	5	11	0	0	0	0	0	0	0	0	0	0	0
1:1500	Bact. para. A serum, 1:50	0	0	0		0	0	0	0	0	0	0	0	0	3	3	2	3
1:1500	Bact. para. B serum, 1:50	4	3	4	11	4	12	0	0	0	3	1	1	1	1	1	1	7
1:1000	Formalin Salt solution	0	0	0	0	0	0								0	0	0	0
1:1000	Formalin Salt solution	0	3	2	5	0	0	0	0	0	0	0	0	0	0	0	0	0
1:3500	Polyval. dysentery serum, 1:100	5	5	5	15	3	3	4	10	3	3	1	7	5	5	5	15	5
1:3500	Polyval. dysentery serum, 1:50	5	5	5	15	4	4	12	4	5	4	13	5	5	5	5	15	5
1:1000	Dysentery Shiga serum, 1:50	0	0	0	0	0	0	0	0	2	0	1	3	0	0	3	3	2
1:1000	Dysentery Shiga serum, 1:50	3	0	1	4	0	0	0	3	3	3	9	1	2	3	6	6	1
1:1000	Normal horse serum, 1:50	2	0	0	2	1	2	5	0	0	3	3	5	5	5	15	5	5
1:1000	Normal horse serum, 1:50	4	3	2	9	3	1	7	5	3	4	12	5	5	15	5	5	5
Control.....	Formalin Salt solution	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Control.....	Formalin Salt solution	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Key: 5, complete; 4, almost complete; 3, weak; 2, very weak; 1, trace; 0, negative agglutination.

*Bact. paratyphosum* B. Formalinized *Bact. typhosum* serum (1:200), practically did not show the pseudo agglutination; most cases were negative (with the exception of no. 1 strain, which had strong agglutination with this serum, and weak agglutination with homologous serum; a peculiar strain but nevertheless typical *Bact. paratyphosum* B), while showing strong agglutination without formalin.

*Bact. paratyphosum* A serum (1:50); pseudo agglutination is shown to be negative with formalin, but strong agglutination occurs without formalin; hence, this serum does not have a tendency to pseudo agglutination with *Bact. paratyphosum* B, if used with formalin.

Polyvalent dysentery serum (1:100), or normal horse serum (1:50), shows negative or weak pseudo agglutination with formalin, and strong agglutination without it.

*Bact. dysenteriae* Shiga serum; many strains are negative or show a weak reaction with formalin, but show more pseudo agglutination without formalin. This, however, is a strongly concentrated serum (1:50), if it is diluted 1:100, it does not agglutinate with *Bact. paratyphosum* B.

*Bact. coli*. As a rule *Bact. typhosum* serum (from immune rabbit or sheep), with or without formalin, shows no agglutination in high concentrations, as 1:200.

*Bact. paratyphosum* A and B serum (from immune rabbit), generally showed no agglutination with *Bact. coli*.

Polyvalent dysentery serum showed strong agglutination with *Bact. coli* either with or without formalin, almost equal to that of the *Bact. dysenteriae* group, as observed by Kligler. This serum we could use for a diagnostic test for *Bact. coli*.

*Bact. dysenteriae* Shiga serum (1:50), showed slight or negative agglutination with formalin, but more without formalin; it is however not a marked reaction.

*Bact. dysenteriae* group. With *Bact. typhosum* serum (1:200), and *Bact. paratyphosum* B serum (1:50), with or without formalin, pseudo agglutination was generally negative, but with *Bact. paratyphosum* A serum 1:50 (obtained from rabbit), there was slight agglutination.

*Bact. dysenteriae* Shiga serum (1:50), shows slight agglutination with the Flexner and Strong strains; but the reaction is stronger with formalin than without; with the Hiss strain, there is no agglutination either with or without formalin. This serum does not have a tendency to group agglutination as do sera of other *Bact. dysenteriae* groups, and horse serum serves for agglutination as well as specific serum.

HORSE SERUM AGGLUTINATION FOR BACT. DYSENTERIAE GROUP  
AND BACT. COLI. (TABLE 5)

Lentz, Park and Williams have found that *Bact. dysenteriae* is agglutinated by normal horse serum, and Gasiakoski, Park and Williams found that *Bact. coli* is agglutinated by normal horse serum. In my experiments nearly every strain of *Bact. dysenteriae* and *Bact. coli* was readily agglutinated by normal horse serum. All samples of horse serum employed, as shown in the table, produced agglutination of both groups of bacilli; only one strain of *Bact. dysenteriae* Shiga no. T obtained from Dr. Shiga, showed negative or weakly positive agglutination.

I have also found that the sera of horses immunized with different bacteria as for instance *Corynebact. diphtheriae*, the meningococcus, *Bact. paratyphosum* and the pneumococcus, have the same agglutinating power as normal horse serum.

COMPARATIVE AGGLUTINATION TESTS WITH 0.7 PER CENT ACID,  
NEUTRAL, AND 0.3 PER CENT ALKALINE BROTH  
CULTURE MEDIA (TABLE 6)

*Bact. typhosum*. Freshly prepared acid broth was used; a heavy growth and actively motile organisms with a tendency to spontaneous agglutination were obtained within 20 hours; with formalin, spontaneous agglutination was prevented almost entirely, while without formalin considerable spontaneous agglutination occurred. These cultures were more easily agglutinated with specific serum than cultures grown in neutral or alkaline broth. According to Dreyer, Eisenberg and Volk, Joos, and Weiss acid increases the agglutinating power.

TABLE 5

*Agglutination of Bact. dysenteriae and Bact. coli, with normal and immunized horse serum with different organisms*

ORGANISM.....			BACT. DYSENTERIAE						BACT. COLI					
			Zhiga No 1	Zhiga No T	Flexner No. 1	Flexner No 2	Strong	Hiss	Number 1	Number 2	Number 4	Number 6	Number 7	
Normal and Immunized sera of Horse	Normal horse	No. 2 {	1:200	5	1	5	5	4	5	5	5	5	5	3
			1:1000	5	0	2	2	0	1	3	3	5	2	1
		No. 3 {	1:200	5	3	3	3	1	4	4	5	1	3	3
			1:1000	5	1	1	1	0	1	1	2	0	0	0
	Anti-meningococcus	No. 6 {	1:200	5	2	5	4	2	5	5	5	5	5	4
			1:1000	5	0	1	0	0	1	5	4	3	2	1
		No. 7 {	1:200	5	0	5	5	5	5	5	5	5	5	4
			1:1000	5	0	4	2	5	2	3	1	3	2	1
	Anti-diphtheria	No. 8 {	1:200	5	0	5	5	4	5	5	5	5	2	4
			1:1000	5	0	2	1	1	5	5	2	3	0	1
		No. 9 {	1:200	5	0	5	5	5	5	5	4	5	3	4
			1:1000	5	0	3	1	2	3	3	2	2	1	1
	Anti-pneumococcus		1:200	5	0	5	5	5	5	5	4	5	4	4
			1:1000	5	0	3	2	1	4	4	1	3	1	1
Anti-B. paratyphosum A		1:200	5	4	5	4	5	5	5	5	5	4	3	
		1:1000	5	1	5	1	4	3	5	5	5	1	1	
Control.....			0	0	0	0	0	0	0	0	0	0	0	

Key: 5, complete; 4, almost complete; 3, weak; 2, very weak; 1, trace; 0, negative agglutination.

In neutral broth the motility, spontaneous agglutination and also specific serum agglutination, were much weaker than in acidified broth. Formalin, prevented spontaneous agglutination entirely.

In alkaline broth most strains of *Bact. typhosum* showed a much weaker growth, motility and spontaneous agglutination.





The specific serum agglutination in this medium was also weak. Therefore the use of alkaline media for *Bact. typhosum* is not favorable for agglutination tests. According to Park and Williams, and Tarchitte, the agglutinating power of the organism is lost in an alkaline medium.

*Bact. paratyphosum A.* These cultures were grown in acid, neutral and in alkaline broth, the reactions of the media being the same as with *Bact. typhosum*.

*Bact. paratyphosum B.* In general the differences in growth and motility in acid, neutral and in alkaline broth were far less marked than in the case of *Bact. typhosum* and *Bact. paratyphosum A.* Both spontaneous and specific agglutination were strongest in the acid medium, but the differences were less than with *Bact. typhosum* or *Bact. paratyphosum A.* However, there is some variation depending upon each strain.

*Bact. dysenteriae* and *Bact. coli* group. On an average the specific serum agglutination test was slightly stronger in acid broth; otherwise the results were almost the same for acid, neutral, and alkaline broth cultures.

In the preceding experiments the nutrient broth was prepared and titrated with phenolphthalein, just before using, as I have reported. The medium becomes more acid on standing, due to the absorption of carbon dioxide from the atmosphere.

#### COMPARATIVE AGGLUTINATION TEST WITH AGAR CULTURE EMULSIFIED IN SALT SOLUTION OR BROTH MEDIUM

Bass and Watkins, Buxton and Vaughan, Kolle, Jordan, Park and Williams, Ritchie, Weil, Wretoski and other workers have used salt solution, for emulsifying the agar culture for the Widal test.

Block, Grumbaum, Durham, and Ker used broth medium with the agar cultures; Hiss and Zinsser used agar cultures emulsified in salt solution or broth medium.

In my experiments, I have not found any particular difference between these fluids. But agglutination is slightly stronger with broth emulsions of *Bact. typhosum*, *Bact. paratyphosum A.*, *Bact. dysenteriae* and *Bact. coli*; with *Bact. paratyphosum B.* the reactions are about equal.



There is slightly less tendency to spontaneous agglutination in a broth medium than in salt solution; therefore, I have concluded that salt is better for preparing suspensions with agar cultures for Widal test.

#### EFFECT OF SODIUM CHLORID ON AGGLUTINATION TESTS (TABLE 7)

Malvoz used agar cultures emulsified with distilled water to avoid chemical changes resulting from sodium chlorid. Weil, working with agar cultures, obtained the same agglutinating reaction, when using either distilled water, or 0.85 per cent sodium chlorid. Asakawa, Bordet, Chick, Joos, Jordan, and Porges claim that sodium chlorid is necessary for the agglutination test. Dreyer, Krumbaar and Smith used tap water in diluting the serum for broth cultures and the results were better than with sodium chlorid. Chick claims 0.42 per cent sodium chlorid gave good results.

In my observation 24 hour agar cultures of *Bact. typhosum*, *Bact. paratyphosum* A and B, *Bact. dysenteriae* and *Bact. coli* emulsified with distilled water were agglutinated by specific serum in dilutions of 1:40 or 1:50, as well as when emulsified with 0.85 per cent salt solution; in this case a certain amount of salt contained in the serum aided agglutination. In weak dilutions of 1:80 or more, there was no agglutination in distilled water (with one exception), but marked agglutination in salt solution. Evidently at that dilution, the salt content of the serum was not sufficient. Only one strain of *Bact. dysenteriae* Flexner no. 2, showed constant agglutination in distilled water with a specific serum dilution of 1:80.

Spontaneous agglutination appeared with spontaneous agglutinating bacilli in almost every instance, when using either distilled water or salt solution.

*Quantity of sodium chlorid.* I have tried various dilutions of salt, varying from 0.05 to 5 per cent, with agar cultures, but no apparent differences were observed in their effect on the agglutination reaction.

It is my conclusion, that only a trace of sodium chlorid is necessary for the Widal test. Since strong solutions are of no advantage, I believe ordinary physiological 0.85 per cent salt is

TABLE 7  
*Comparative agglutination tests of agar cultures (37°C.—twenty-four hours), emulsified with 0.85 per cent salt solution, and distilled water*

ORGANISM.....	BACT. TYPHOSEUM			BACT. PARA A			BACT. PARA B			BACT. COLI			BACT. DYSENTERIAE						
	No. 1	No. 2	No. 7	No. 1	No. 2	Nos. 4, 2	No. 2	Nos. 3, 2	Nos. 4, 2	No. 4	No. 6	Nos. 1, 2	Nos. 2, 2	Shiga T	Fleischer 1	Fleischer 2	Strom	Hiss	
Number of strain.....	No. 1	No. 2	No. 7	No. 1	No. 2	Nos. 4, 2	No. 2	Nos. 3, 2	Nos. 4, 2	No. 4	No. 6	Nos. 1, 2	Nos. 2, 2	Shiga T	Fleischer 1	Fleischer 2	Strom	Hiss	
Spontaneous agglutination on cultures.....	0	0	2	0	0	2	2	2	2	0	0	2	2	0	0	0	0	0	0
Dilution of specific serum	1:40		1:80	Water		Salt solution		Water		Salt solution		Water		Salt solution		Water		Salt solution	
	5	5		5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Control.....	0	0	1	0	0	1	0	0	1	0	0	1	1	0	0	0	0	0	0

Key: 5, complete; 4, almost complete; 3, weak; 2, very weak; 1, trace; 0, negative agglutination.

quite as satisfactory for agar or broth cultures, especially as it is to be found in most laboratories.

There were no indications that the use of tap water or distilled water was better than salt solution, when we used broth cultures.

COMPARATIVE AGGLUTINATION TESTS WITH VARIOUS CULTURE MEDIA (TABLE 8)

In conducting agglutination tests of the colon-typhoid group various kinds of culture media have been used. The cultures were grown at 37°C. for 24 hours in neutral broth, one per cent glucose broth, one per cent pepton water and on agar, the agar cultures being emulsified with salt solution.

*Bact. typhosum.* Grown in plain broth, glucose broth, pepton water and on agar, it showed the strongest agglutination with specific serum in the first named medium, becoming weaker in each of the others in the order given; the reaction was very weak with agar cultures. Dreyer claims that glucose broth cultures have a tendency to spontaneous agglutination, but my results in using glucose broth were quite irregular; spontaneous agglutination occurred in some cases, while in others there was no spontaneous agglutination, but there was generally negative or weak spontaneous agglutination with both spontaneous and non-spontaneous agglutinating types.

As compared with cultures in plain broth those grown in glucose broth showed very irregular and weak motility. Formalized serum showed stronger agglutination than plain serum; especially marked was the difference with glucose broth. With plain broth or pepton water or agar cultures, the difference was a great deal less marked.

*Bact. paratyphosum A.* Cultures grown in glucose broth showed weak motility, many strains agglutinating spontaneously, even though non-spontaneous agglutinating bacilli were used. Formalin does not prevent this agglutination.

In specific agglutination tests glucose broth cultures showed considerable reaction, even more than with pepton water or agar cultures. However, these results are due to the spontaneous agglutination in the glucose broth. It is evident, that this

TABLE 8  
 Comparative agglutination tests, with neutral broth, 1 per cent glucose broth, 1 per cent pepton water, and agar  
 (in salt solution) 37°C.—twenty-four hours culture, Added O, 1 per cent formalin

CULTURE MEDIA.....	No. 1				No. 2				No. 3				No. 4				No. 6				TOTAL				
	PLAIN BROTH	1 PER CENT GLUCOSE BROTH	1 PER CENT PEPTON WATER	AGAR	PLAIN BROTH	1 PER CENT GLUCOSE BROTH	1 PER CENT PEPTON WATER	AGAR	PLAIN BROTH	1 PER CENT GLUCOSE BROTH	1 PER CENT PEPTON WATER	AGAR	PLAIN BROTH	1 PER CENT GLUCOSE BROTH	1 PER CENT PEPTON WATER	AGAR	PLAIN BROTH	1 PER CENT GLUCOSE BROTH	1 PER CENT PEPTON WATER	AGAR	PLAIN BROTH	1 PER CENT GLUCOSE BROTH	1 PER CENT PEPTON WATER	AGAR	
Bact. typhosum.....	+	<+	+	+	+	<+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility.....																									
Spontaneous agglutination on culture.....	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Bact. typhosum { 1:25,000	5	4	3	2	5	4	4	2	5	4	3	2	5	4	1	2	5	4	1	2	20	16	14	8	
serum..... { 1:100,000	3	2	1	0	2	2	1	0	3	2	0	0	3	2	1	1	3	2	1	1	14	6	4	1	
Control.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Bact. para A.....																									
Motility.....	+	<+	+	+	+	<+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Spontaneous agglutination on culture.....	0	2	2	0	0	1	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Bact. para A serum.. { 1:10,000	5	5	4	2	4	4	4	2	5	5	1	2	5	5	4	4	5	4	4	4	19	19	16	10	
..... { 1:50,000	2	3	2	0	1	1	0	0	1	3	1	0	2	1	1	0	2	1	1	0	6	8	4	0	
Control.....	0	2	1	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	5	1	0	

	No. 1			No. 2			No. 3			No. 4			4 strains			
	+	<+	+	+	<+	<+	+	<+	+	+	<+	<+	+	<+	<+	
Bact. para B.....																
Motility.....																
Spontaneous agglutination on culture.....																
Bact. para B serum. {	0	2	1	0	0	1	1	1	0	1	0	1	0	3	2	0
1:2,000	3	4	3	3	4	5	3	3	3	3	3	2	4	4	4	3
1:5,000	2	3	2	2	2	3	3	1	2	2	1	0	2	4	3	2
Control.....	0	3	0	0	0	2	1	0	0	1	0	0	0	2	1	0
Bact. coli.....																
Motility.....																
Spontaneous agglutination on culture.....																
Horse serum. {	0	2	1	0	0	2	1	1	0	2	1	0	0	2	0	0
1:700	5	5	4	2	5	5	4	2	5	5	5	3	3	3	2	2
1:30,000	2	3	2	2	2	3	4	2	2	2	2	1	0	1	1	0
Control.....	0	2	0	0	0	3	3	0	0	2	0	0	0	1	1	0
Bact. dysenteriae.....																
Motility.....																
Spontaneous agglutination on culture.....																
Polyv. B. dysenteriae {	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1:2,000	5	5	5	5	5	3	4	4	5	4	4	4	5	3	5	4
1:15,000	4	3	4	4	2	0	2	1	2	1	2	1	2	0	2	2
Control.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Key: +, active; <+, slow; <<+, very slow motility; 5, complete; 4, almost complete; 3, weak; 2, very weak; 1 trace; 0, negative agglutination.

medium is not suitable for the agglutination test for *Bact. paratyphosum* A.

Occasionally spontaneous agglutination in pepton water cultures is difficult to prevent, while in plain broth it is relatively easy to prevent; in pepton water there is but a slight tendency to spontaneous agglutination even with non-spontaneous agglutinating bacilli. Specific agglutinations with pepton water cultures were slightly weaker than with plain or glucose broth cultures. With formalin the reaction was slightly stronger than without.

With agar cultures the agglutination reaction was generally slow and weak and appeared somewhat incomplete, depending however upon the strain and age of the culture. With or without formalin almost the same results are obtained with non-spontaneous agglutinating bacilli.

*Bact. paratyphosum* B. Glucose broth cultures of *Bact. paratyphosum* B usually show weak motility and strong spontaneous agglutination, which is not prevented when treated with formalin; even non-spontaneous agglutinating bacilli give the same result. This medium does not appear to be satisfactory for the Widal test with *Bact. paratyphosum* B.

Cultures in plain broth and pepton water showed more vigorous motility and almost the same agglutinating reaction with specific serum. But in pepton water with non-spontaneous agglutinating bacilli, there was a slight tendency to spontaneous agglutination even when the cultures were mixed with salt solution or with formalin. It appears that plain broth cultures are more reliable than are those in pepton water.

The agar cultures showed weak specific agglutination in comparison with the other three kinds of media, but did not have the tendency to spontaneous agglutination.

*Bact. coli*. Glucose broth cultures of most strains of *Bact. coli* grown for eighteen to twenty-four hours at 37°C. showed considerable spontaneous agglutination with both spontaneous and non-spontaneous agglutinating bacilli; hence this medium cannot be used for the specific agglutination test.

Certain strains in pepton water cultures showed a slight tendency to spontaneous agglutination, while in broth they showed no spontaneous agglutination. The specific agglutinations were weaker in pepton water than broth medium; therefore I consider the latter most suitable for the Widal test with *Bact. coli*.

Agar cultures show very weak agglutination, but there is no tendency to spontaneous agglutination with *Bact. coli*.

*Bact. dysenteriae* group. This group showed strong specific agglutination in plain broth cultures, it was slightly weaker in pepton water and agar and especially weak in glucose broth; but glucose broth did not show spontaneous agglutination.

TABLE 9

Relation of age to specific agglutinating power with non-spontaneous agglutinating bacilli, grown on broth culture medium

ORGANISM .....	BACT. TYPHOSUM				BACT. PARA A				BACT. DYSENTERIAE						
	No. 1	No. 2	No. 3	Total 3	No. 1	No. 2	No. 3	Total 3	Shiga No. 1	Flexner	Strong	Hiss	Total 4	Shiga No. T	
Number of strain.....															
Ages of cultures and specific serum	1 day	5	5	5	15	5	5	5	15	5	5	5	5	20	2
	2 days	5	4	5	14	5	4	4	13	5	5	5	3	18	3
	3 days	4	3	4	11	1	3	3	10	4	4	4	2	14	4
	5 days	3	2	3	8	3	2	2	7	3	3	2	1	9	5

Key: 5, complete; 4, almost complete; 3, weak; 2, very weak; 1, trace; 0, negative agglutination.

#### RELATION OF AGGLUTINATION POWER TO AGE OF CULTURES (TABLE 9)

Dreyer claims that cultures more than one day old had a weak agglutinating power with specific serum. My experiments for a period of five days, with *Bact. typhosum*, *Bact. paratyphosum* A and the *Bact. dysenteriae* group, indicated that cultures on neutral broth medium one day old were agglutinated best, the reactions become weaker each day thereafter. However, I found that one strain of *Bact. dysenteriae* Shiga no. T showed just the opposite result, the agglutinating power becoming stronger with older cultures over a period of five days.

SPONTANEOUS AGGLUTINATION ACCORDING TO AGE OF CULTURES  
(TABLE 10)

When spontaneous agglutinating bacilli were grown in neutral broth or pepton water, spontaneous agglutination was more vigorous when cultures were but one day old, diminishing steadily afterwards and almost disappearing on the fifth day.

I concluded therefore that both spontaneous and specific agglutinating power diminish with the age of most strains of the colon-typhoid group.

TABLE 10

*Spontaneous agglutination according to age of bacilli growing in plain broth at 37° C.*

ORGANISM.....	BACT. TYPHOSUM			BACT. PARA A			BACT. PARA B			TOTAL
	1	2	3	1	2	3	1	2	3	
Number of strain.....	1	1	1	1	1	1	1	1	1	9
Ages of cultures.....	1 day	3	3	3	3	2	3	3	3	26
	2 days	2	2	2	2	2	2	2	3	19
	3 days	1	1	2	1	1	1	1	2	11
	5 days	0	0	1	0	0	0	0	1	2

*Key:* 3, strong; 2, medium; 1, weak spontaneous agglutination.

COMPARATIVE RESULTS OF MICROSCOPIC AND MACROSCOPIC AGGLUTINATION TESTS

Chick, Dreyer, Garrow, Jordan, Panton, Walker, Wilson, and several other investigators prefer the macroscopic method; Delepine, Ritchie, and several other workers have used the microscopic method.

In using non-spontaneous agglutinating cultures, strong agglutination could be seen equally well with either method, and weak agglutination better by the microscopic method. For instance, in some cases which appeared negative macroscopically, small clumps could be seen microscopically when examined at the end of three hours. These small clumps are of great value in certain cases of very weak reactions. This is especially noticeable with the *Bact. dysenteriae* group, but less so for



*Bact. typhosum*, *Bact. paratyphosum* A and B, and the *Bact. coli* group.

In the case of *Bact. typhosum* and *Bact. paratyphosum* A and B, spontaneous agglutination generally appears weak macroscopically and strong microscopically; but with *Bact. coli* it appears to be almost the same with either method. It is specially to be noted that when using spontaneous agglutinating bacilli for the Widal test, the macroscopic method was found more reliable.

For the fundamental or graphic agglutination test, I used non-spontaneous agglutinating bacilli. When agglutination was

TABLE II  
*Agglutination tests at different temperatures*

ORGANISM .....	BACT. TYPHOSUM				BACT. PARA A			BACT. PARA B			BACT. COLI			BACT. DYSENTERIAE							
	No. 1	No. 2	No. 3	Total 3	No. 1	No. 2	No. 3	Total 3	No. 1	No. 2	No. 3	Total 3	No. 1	No. 2	No. 3	Total 3	Shiga	Flexner	H. ss.	Total 3	
Number of strain .....																					
With each specific serum	Room temperature.....	5	5	5	15	5	5	5	15	5	5	5	15	5	5	5	15	5	5	5	15
	37°C. water bath	5	5	5	15	5	5	5	15	5	5	5	15	5	5	5	15	5	5	5	15
	45°C. water bath	4	4	4	12	4	4	4	12	4	4	4	12	4	4	4	12	4	4	4	12
	55°C. water bath	3	3	2	8	4	4	3	11	3	3	3	9	4	3	3	10	3	3	3	9
Controls of all	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

Key: 5, complete; 4, almost complete; 3, weak; 2, very weak; 1, trace; 0, negative agglutination.

weak or the case questionable, I resorted to the microscopic method for final determination.

#### AGGLUTINATION AT DIFFERENT TEMPERATURES (TABLE 11)

Delepine, Jordan, Konrich, Lion, Meyer and Kilgore, Widal, and many others conducted agglutination tests at 37°C.; Dreyer and Blake, Hetsch, Kolle, Kutscher, Porges, and Weil advise the use of 50°C. to 55°C.; Joos 35°C. to 40°C.; Berliner and Cohn, and Durham recommend room temperature.

Recently many workers have read the test, after keeping the tubes in the water bath two to three hours at from 50°C. to 55°C. then leaving them at room temperature or in the cold room over night.

In my observations on the effect of temperature of the agglutination with *Bact. typhosum*, *Bact. paratyphosum* A and B, *Bact. coli*, and the dysentery group, the tubes kept in a water bath for two hours at 45°C., showed a slightly weaker reaction than those kept at room temperature or 37°C., while at 55°C., the reaction was much weaker than at 45°C. The results, after leaving the tubes for 2 to 3 hours, or even for twenty-four hours at room temperature, were just the same as they were when the tubes were placed in the water bath at 37°C. for two to three hours and then left standing at room temperature over night.

TABLE 12

*Agglutination tests conducted with bacilli after heating at 55°C. for two hours*

ORGANISM .....	BACT. TYPHOSUM			BACT. PARA A			BACT. PARA B			BACT. COLI			BACT. DYSENTERIAE				
	No. 1	No. 2	Total 3	No. 1	No. 2	Total 3	No. 1	No. 2	Total 3	No. 1	No. 2	Total 3	Shiga	Flexner	His	Total 3	
Number of strain .....																	
With specific serum.....	Unheated			55°C. 2 hours													
	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	3	3	3	9	3	2	2	7	3	4	3	10	3	4	3	10	2
Control of both.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

*Key:* 5, complete; 4, almost complete; 3, weak; 2, very weak; 0, negative agglutination.

In the next experiments (table 12), bacilli were heated at 55°C. for two hours before conducting the agglutination test; it became evident that these bacilli had less agglutinating power than the unheated bacilli. On the other hand, the serum which had been heated at 55°C. for two hours gave just the same results as serum which had not been heated.

When temperatures from 45°C. to 55°C. were used, the clumps were smaller than when the test was conducted at room temperature or 37°C. This was true when either the microscopic or macroscopic methods were employed.

The latter temperature is therefore to be preferred, with the colon-typhoid group with or without formalin; the end results, in relation to the temperature, are just the same.

## THE TIME FACTOR IN THE AGGLUTINATION TEST

Agglutination tests after various periods of time were carried out with the colon-typhoid group, in relation to certain titres of the serum.

*Bact. typhosum* showed the reaction quicker than the other organisms, but the agglutination reaction of most of the strains was completed only after standing over night, instead of after three hours. However, some strains showed almost complete agglutination in three hours; others only a trace in this time. Of course with strong serum, agglutination took place soon after mixing with the bacterial suspension.

With *Bact. paratyphosum* B, the results were of the same order, but less rapid than with *Bact. typhosum*.

*Bact. coli* comes after *Bact. paratyphosum* B, with regard to the speed of the reaction.

*Bact. paratyphosum* A, and *Bact. dysenteriae* show relatively very slow agglutination; in most instances, the reaction was definitely obtained only after standing over night.

However, the speed of the reaction varies with the strains of bacilli; some strains were always quickly agglutinated and others more slowly.

## THE USE OF SEVERAL STRAINS FOR THE WIDAL TEST (TABLE 13)

In the Widal test most bacteriologists use only one strain of bacillus with the serum of the patient. This method does not give reliable results. Ordinarily we use polyvalent immune serum for the identification of a bacillus; for the same reason we should use several strains in testing the serum of a patient.

A serum agglutination reaction depends upon the organism infecting the patient. If the serum agglutinates the stock culture, it indicates a close relationship between this organism and the organism which infected the patient; a negative test indicates distant or no relationship.

In a case of typhoid fever (P. B. G. Hospital) the serum showed positive agglutination for three strains (nos. 1, 4, 33), but negative for three others (nos. 3, 7, 29), and doubtful for one more (no. 2).

In performing this test, therefore, I have found it convenient to grow each strain (6 or more) of this organism in broth medium at 37°C., for eighteen to twenty hours. Formalin 0.1 per cent is added to each of the cultures, which are then all mixed together, and are ready for immediate use; or they may be stored in a cold room until needed, as in Dreyer's method. This preparation gives reliable results in diagnosis with an unknown serum.

TABLE 13  
*The serum of typhoid fever, Widal test with different strains*

		BACT. TYPHOSUM 7 STRAINS						
		No. 1	No. 4	No. 33	No. 2	No. 3	No. 7	No. 29
Serum dilutions of typhoid fever	1:25	5	5	5	3	1	0	3
	1:50	5	4	4	2	0	0	1
	1:100	4	3	3	1	0	0	0
	1:150	3	2	1	0	0	0	0
	1:200	1	0	0	0	0	0	0
Results.....		+	+	+	?	0	0	0

*Key:* 5, complete; 4, almost complete; 3, weak; 2, very weak; 1, trace; 0, negative agglutination; +, positive reaction.

#### SUMMARY

1. Formalin 0.05 to 0.2 per cent prevents spontaneous agglutination almost entirely in some instances and entirely in others with *Bact. typhosum* and *Bact. paratyphosum* A and in a lesser degree with *Bact. paratyphosum* B; with *Bact. coli* formalin has no effect in this regard.

2. One-tenth per cent formalin prevents spontaneous agglutination and increases specific agglutination, having a strong effect on *Bact. typhosum* and *Bact. paratyphosum* A, but less on *Bact. paratyphosum* B and *Bact. dysenteriae* and none on *Bact. coli*.

3. Formalin prevents pseudo agglutination to a great extent in cross agglutination test with different sera.

4. Cultures in acid medium in which there is heavy growth and active motility, yield stronger agglutination than cultures

in neutral broth with *Bact. typhosum* and *Bact. paratyphosum* A; this effect is less noticeable in cultures of *Bact. paratyphosum* B, *Bact. dysenteriae* and *Bact. coli*, but the agglutinating power of these organisms is very weak in alkaline media. Alkaline media are therefore not suitable for the agglutination test with cultures of these bacteria.

5. Agar cultures emulsified with 0.85 per cent salt solution or neutral broth are almost equal in susceptibility to agglutination, but certain strains have a tendency to spontaneous agglutination in broth medium; therefore salt solution is better.

6. As to the effect of sodium chlorid on agglutination reactions I could find no difference in the degree of agglutination reaction when using strong or weak solutions of sodium chlorid; only traces are necessary for agglutination.

7. Glucose broth cultures of *Bact. paratyphosum* A and B and *Bact. coli* showed a tendency to spontaneous agglutination; *Bact. typhosum* showed weaker specific agglutination in glucose broth, but stronger than in pepton water, only occasionally showing spontaneous agglutination. The dysentery group yielded weaker agglutination with glucose broth cultures than with plain broth, pepton water and agar cultures.

Pepton water cultures in general show a slight tendency to spontaneous agglutination, when non-spontaneous agglutinating bacilli are grown therein. This spontaneous agglutination is difficult to prevent even when formalin is used and the specific agglutination reactions of *Bact. typhosum*, *Bact. paratyphosum* A and B and *Bact. coli* are weaker than in plain broth or glucose broth. On the other hand the dysentery group yielded stronger specific agglutination reactions in this medium than in glucose broth.

Agar cultures showed much weaker agglutination than broth and pepton water cultures.

In general, plain broth cultures gave the best results.

8. Susceptibility of most cultures to agglutination decreases with age; eighteen to twenty-four hours cultures were found best suited for agglutination tests.

9. The microscopic is more reliable than the macroscopic method for weak or graphical agglutination work; with strongly agglutinating sera showing the same reactions in both, the macroscopic method is preferred, because it is easier and quicker than the microscopic method. Therefore the macroscopic method is to be regarded as most satisfactory for usual work and particularly when many reactions are to be performed.

10. Weak agglutination results if bacilli are heated at a temperature of 45 to 55°C. for two to three hours; this temperature does not affect the agglutinating activity of serum. Room temperature and 37°C., both gave better results than higher temperatures for the incubation of tests.

11. Two to three hours are not enough for complete agglutination; the tests should be set aside and read the next day.

12. Normal horse serum showed strong agglutination reaction with *Bact. dysenteriae*, *Bact. coli* and other bacilli of the typhoid-colon group.

Polyvalent *Bact. dysenteriae* serum had strong agglutinating power for *Bact. typhosum* and *Bact. coli* as well as for the dysentery group.

13. In serum diagnosis with the Widal test, many different strains should be used since from one strain alone, reliable results cannot be obtained.

#### SUPPLEMENTARY NOTES

*a.* Eighteen to twenty-four hours cultures of the majority of strains of *Bact. coli* grown at 37°C., show a tendency to spontaneous agglutination in most culture media; cultures grown at room temperature show less and are therefore preferable.

*b.* Old stock cultures transplanted to a fresh culture medium at first show weak, or no, susceptibility to agglutination; however, after a few sub-cultures (once or twice a day, if actively growing bacilli), they regain this property. The same holds good for all cultures of new bacilli, and it is necessary therefore, for accurate agglutination work to test the agglutinability of the bacterial culture with a control serum of known agglutinating powers.

c. Most cultures grown at 37°C. for eighteen to twenty-four hours show a slight pellicle on the surface; to avoid mistaking this for spontaneous agglutination the cultures should be shaken and left at room temperature for a while, after the pellicle falls to the bottom of the test tube.

d. Heavy growths yield better results in the Widal test when diluted 2 or 4 times with salt solution.

e. Acid broth (0.2 per cent) cultures should be used for routine agglutination tests inasmuch as acidity is useful and greatly helps in weak agglutination.

f. It is well known that commercial formalin contains formic acid. Fearing that the formic acid would interfere with the Widal reaction, I have tried certain strains of *Bact. typhosum* and *Bact. paratyphosum* group with various dilutions of formic acid (0.1 to 10 per cent) and found that with these dilutions weak chemical agglutinations are produced, but the specific agglutination is diminished. With a weak solution of formalin (0.1 to 1 per cent) no chemical agglutination is produced and spontaneous agglutination is prevented, as above stated. Evidently the quantity of formic acid in these concentrations of formalin is so small that it does not interfere with the reaction. Commercial formalin gave satisfactory results throughout this work.

g. The addition of phenol (0.1 per cent) or corrosive sublimate (0.01 per cent) did not change normal agglutination power. Tricresol (0.1 per cent) slightly weakened agglutination in the colon-typhoid group. All substances produced weak or negative agglutination reactions when used in more concentrated solutions than those above mentioned.

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# A STUDY OF SPONTANEOUS AGGLUTINATION IN THE COLON-TYPHOID GROUP OF BACILLI

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It is well recognized that spontaneous agglutination may occur in broth cultures of *Bact. typhosum* and *Bact. paratyphosum* A and B. Because of this phenomenon, the results of agglutination tests with specific sera are not reliable under such conditions, since it is practically impossible to distinguish between spontaneous and specific agglutination.

Block in 1897 found that cultures which were transplanted too frequently, may agglutinate spontaneously. He further observed that when cultures were grown in alkaline broth the same phenomenon occurred. Delepine and Fison (1897) also noted spontaneous agglutination in cultures of *Bact. typhosum*. Kruse, Rittershaus, Kemp and Metz described spontaneous agglutination of typhoid and dysentery bacilli in plain broth cultures and in very concentrated peptone broth cultures. Nicolle (1898) reported that changes in the bacterial protein occurred in old cultures, and that the organisms became more sensitive and readily agglutinated spontaneously in such cultures. Smith and Reagh (1903) reported that the *Bact. icteroides* developed granular colonies on gelatin cultures; i.e. were agglutinated spontaneously. Steinhardt (1904) found that in agar cultures, showing spontaneous agglutination, the colonies were irregular in shape and less translucent than the colonies of the type which did not agglutinate spontaneously. Teague and McWilliams (1917) reported that organisms isolated from the blood of a rabbit which had been injected with *Bact. typhosum*,

produced different kinds of colonies when grown on agar plates, some being opaque and others transparent; in addition there were also differences in their contours, some having irregular outlines while others were round with smooth edges. Furthermore some colonies were small and others large.

#### APPEARANCE OF SPONTANEOUSLY AGGLUTINATED COLONIES ON AGAR PLATES

Plating many stock cultures upon two per cent agar plates we have found two distinct types of colonies. The first of these presents a smooth surface and a regular outline, and is not spontaneously agglutinated. The other type shows a much heavier growth, has a rough surface, is irregularly round, with a serrate border and is much more transparent, both at its center and border, than the first type of colony. That most of the colonies of this second type undergo spontaneous agglutination, can be noted by means of the low power microscope or hand lens. In some instances, upon sub-culturing, certain strains of those colonies which previously had been transparent became opaque and vice versa; or an organism originally producing small colonies produced large ones. These points of differentiation are not so important as those relating to the outline of the colony and smoothness or roughness of the surface. The available space for growth on the plate as well as the rate of growth, appear to influence these characteristics.

#### SPONTANEOUS AGGLUTINATION IN BROTH CULTURES (TABLE 1)

Steinhardt, Teague and McWilliams found in broth cultures of *Bact. typhosum*, which underwent spontaneous agglutination, flocculi or pellicle formation as contrasted with a uniform, cloudy growth of other organisms. Smith and Reagh showed that a broth culture of *Bact. icteroides* became clear after spontaneous agglutination had occurred.

We have observed spontaneous agglutination of *Bact. typhosum*, *Bact. paratyposum* A and B, *Bact. enteritidis*, *Bact. dysenteriae* and *Bact. coli* in pepton water, glycerol broth and glucose

broth. In many cases the broth was clear macroscopically, but the clumps were visible when a hanging drop was examined. More or less of the precipitate collected in the bottom of the tubes. In some instances flocculi and a pellicle were visible on the surface of the culture, and there was always a precipitation in the bottom, either spontaneous or following agitation of the tube. In other cases, small clumps could be seen with the naked eye scattered throughout the culture. With the typhoid and paratyphoid bacilli there was a uniform cloudy growth and apparently an absence of spontaneous agglutination when

TABLE I

*Variations of growth in broth medium. Cultures spontaneously agglutinated show clear supernatant fluid, precipitation, pellicle, flocculi or small clumps, others appear uniformly cloudy, to the naked eye like cultures not spontaneously agglutinated*

ORGANISMS GROWN IN BROTH	PRECIPITATION, PELLICLE AND FLOCCULI	UNIFORMLY CLOUDY	TOTAL NUMBER OF STRAINS
<i>Bact. typhosum</i> .....	4	12	16
<i>Bact. paratyphosum</i> A.....	7	9	16
<i>Bact. paratyphosum</i> B.....	15	1	16
<i>Bact. coli</i> .....	5	0	5

A tabulation of our experiments shows the following results: *Bact. paratyphosum* B showed precipitation with formation of pellicles and small clumps in clear fluid in all cases with the exception of one strain. *Bact. coli*, 5 strains exhibited this same phenomenon in every case. *Bact. paratyphosum* A showed 7 positive and 9 negative, *Bact. typhosum* 4 positive and 12 negative tests.

examined with the naked eye, but with a hand lens, flocculi were clearly visible in the supernatant fluid.

We have, therefore, been led to conclude that in broth cultures when flocculi are visible, macroscopic observation may be relied upon; but in cloudy cultures, microscopic examination should always be employed.

In twenty-four hour cultures at 37°C., of microorganisms which do not undergo spontaneous agglutination, the growth in pepton water, glycerol or glucose broth, whether the reaction of the media be acid, neutral or alkaline, is generally uniformly cloudy. In some cases, however, there is a pellicle and flocculi are seen near the surface of such cultures.

Microscopic examination of the twenty-four-hour broth cultures by the hanging drop method showed differences in morphology and motility between those organisms which agglutinated spontaneously and those which did not. In most cases the bacilli which undergo spontaneous agglutination are longer, sometimes having the appearance of being fungiform and showing relatively greater motility. The bacilli which do not agglutinate spontaneously are shorter and are less motile.

#### COLONY CHANGES IN ARTIFICIAL CULTURE MEDIA (TABLE 2)

Löffler (1906) found four types of colonies of *Bact. coli*: (1) transparent, (2) flat, (3) thick, and (4) opaque. According to his evidence, these types are constant and do not change. Baerthlein (1911) reported a cholera vibrio colony that did not change after passage through animals, but in a stock culture twenty-two days afterwards, in several subcultures, the yellow type turned light and twenty-eight days later the light colony changed to a yellow one. Steinhardt found spontaneous agglutination in *Bact. typhosum* after the twentieth passage through bactericidal serum cultures. Teague and McWilliams state that they found some changeable bacilli, but they do not draw any definite conclusions from their finding.

According to our observation colonies of many strains of the colon-typhoid group may lose the property of spontaneous agglutination, while others that do not show this property at first may show it after some time.

From a stock culture we obtained colonies which were, and others which were not, agglutinated spontaneously. These were spread on plates of two per cent agar (using very high dilution of the bacteria in broth or salt solution so that each single colony should be separated from every other colony) and incubated for 24 hours at 37°C. The colonies thus grown were inoculated into broth tubes and placed in the incubator. Subcultures were made every two days by inoculating one loopful of the broth culture into a fresh tube of broth. The bacilli were examined after each sub-culture by streaking a loopful of the freshly inoculated broth on agar plates. The following results on agar plates and in cultures were obtained:

TABLE 2

Broth cultures of the isolated colony were kept at 37°C., and transplanted every second day, then examined for any change of colonies by streaking on agar plates

ORGANISM	NUMBER OF STRAINS	TYPE OF COLONY	NUMBER OF DAYS, WHEN CHANGE FOUND, REPEATED ONE TO THREE TIMES		
<i>Bact. typhosum</i> .....	No. 1	Non-spont. aggl.	21	35	
		Spont. aggl.	10	13	
	No. 2	Non-spont. aggl.	6	6	13
		Spont. aggl.	16	14	5
	No. 29	Non-spont. aggl.	32	12	20
		Spont. aggl.	50	48	47
<i>Bact. paratyphosum A</i>	No. 1	Non-spont. aggl.	4	4	3
		Spont. aggl.	4	4	4
	No. 2	Non-spont. aggl.	3	3	3
		Spont. aggl.	-30		
	No. 3	Non-spont. aggl.	7	6	8
		Spont. aggl.	12	18	15
<i>Bact. paratyphosum B</i>	No. 4	Non-spont. aggl.	5	5	4
		Spont. aggl.	13	25	
	No. 5	Non-spont. aggl.	5	10	
		Spont. aggl.	-60		
	No. 6	Non-spont. aggl.	5	8	12
		Spont. aggl.	24	25	
<i>Bact. coli</i> .....	No. 1	Non-spont. aggl.	2	3	3
		Spont. aggl.	2	3	2
	No. 2	Non-spont. aggl.	7	4	
		Spont. aggl.	7	10	
	No. 3	Non-spont. aggl.	10	13	
		Spont. aggl.	30	28	
<i>Bact. enteritidis</i> .....	No. 2	Non-spont. aggl.	24		
		Spont. aggl.	16		

TABLE 2—Continued

ORGANISM	NUMBER OF STRAINS	TYPE OF COLONY	NUMBER OF DAYS, WHEN CHANGE FOUND. REPEATED ONE TO THREE TIMES		
<i>Bact. enteritidis</i> .....	No. 3	Non-spont. aggl.	7		
		Spont. aggl.	5		
	No. 7	Non-spont. aggl.	5		
		Spont. aggl.	5		
<i>Bact. dysenteriae</i> .....	Shiga No. 1	Non-spont. aggl.	22	5	
		Spont. aggl.	4		
	Flexner No. 1	Non-spont. aggl.	8		
		Spont. aggl.	17		

Key, — No change.

(a) Different organisms showed a differentiation into two types of colony at different periods of growth, *Bact. coli* showing a change in from 2 to 30 days.

(b) *Bact. paratyphosum* A showed the change within 3 to 14 days (some strains however, remaining constant even after 30 days).

(c) *Bact. paratyphosum* B showed a transition period of from 4 to 30 days, while some strains did not show the spontaneously agglutinated type of colony even after 60 days.

(d) *Bact. dysenteriae* group showed changes in from 4 to 22 days (some strains being negative after 30 days).

(e) *Bact. enteritidis* changed in from 5 to 24 days.

(f) *Bact. typhosum* showed the longest period, the time varying from 6 to 50 days in some strains, while others did not show changes even after 3 months or more.

From colonies of known type (as determined above) isolated on agar plates, the different members of the colon-typhoid group were inoculated into broth media and allowed to grow at room temperature without sub-culture, plates being made every five or ten days to determine the type of colony. In 33 strains of *Bact. typhosum* of both types, changes were observed to take place in 30 strains in fifteen to ninety days, while three strains,



originally of a spontaneously agglutinating variety, showed no change after one hundred days. Eighteen strains of *Bact. paratyphosum* A showed changes in from ten to eighty-five days, two strains of an agglutinating type, showed no change until after one hundred days. Nineteen strains of *Bact. paratyphosum* B, showed changes in from fifteen to one hundred days, while two strains of spontaneously agglutinating type did not change even in one hundred days. All strains of *Bact. coli* showed a change in from ten to thirty days. *Bact. enteritidis* was examined after 60 days growth, and all 8 strains showed a change. With the *Bact. dysenteriae* group two strains showed changes in thirty-five to forty days, while two strains showed no change after sixty days.

The period at which the change in colony occurs depends upon the strains; some strains always change within a short time and others only after many days.

#### ISOLATION OF TYPES OF COLONY FROM STOCK CULTURES (TABLE 3)

In our first work with stock cultures three types were found. These were, pure colonies not showing spontaneous agglutination, pure colonies showing spontaneous agglutination and mixed colonies showing the presence of both types.

In a study of the colon-typhoid group of bacilli from agar stab cultures which had ordinarily been sub-cultured at intervals of two or three months, there were found many cultures producing colonies of only one kind, namely those which agglutinated spontaneously; other cultures yielded colonies of bacilli all of which failed to agglutinate spontaneously.

In the present work extending over a period of a year, it was found that each pure type after frequent transplantation tended to change into both types; of 29 strains of *Bact. typhosum* showing pure colonies of the spontaneous agglutinating type only 3 strains remained unchanged. Of 4 originally pure strains of *Bact. coli* used only 2 adhered to the original type. *Bact. paratyphosum* A (19 strains) *Bact. enteritidis* (9 strains) and *Bact. dysenteriae* (3 strains) showed both types of colonies. *Bact. paratyphosum* B (21 strains) showed both types of colonies, but

2 strains (nos. 19 and 21), after frequent sub-culture in broth, pepton water and agar throughout a period of one year, gave 1 type of spontaneously agglutinating bacilli during the entire period. When, however, 1 per cent glucose broth was used for daily transplants, culture no. 19 showed 2 types of bacilli after seven days and culture no. 21 after eighteen days.

A new strain of *Bact. typhosum* was isolated from the blood of a patient at the Peter Bent Brigham Hospital in the spring of

TABLE 3  
*Isolation of colonies from stock stab cultures of agar medium*

	ORGANISM	NUMBER OF STRAINS	SPONTANEOUS AGGLUTINATION	NON-SPONTANEOUS AGGLUTINATION	MIXED, SHOWING BOTH TYPES	DOUBTFUL AGGLUTINATION
First isolation	<i>Bact. typhosum</i> .....	33	4	15	13	1
	<i>Bact. paratyphosum A</i> .....	19	5	7	7	
	<i>Bact. paratyphosum B</i> .....	23	4	3	16	
	<i>Bact. coli</i> .....	8	1	3	4	
	<i>Bact. enteritidis</i> ....	9	1	2	6	
	<i>Bact. dysenteriae</i> ...	3	—	1	2	
Isolation of subcultures after several months	<i>Bact. typhosum</i> .....	33	3	—	29	1
	<i>Bact. paratyphosum A</i> .....	19	—	—	19	
	<i>Bact. paratyphosum B</i> .....	25	—	—	23	
	<i>Bact. coli</i> .....	8	—	2	6	
	<i>Bact. enteritidis</i>	9	—	—	9	
	<i>Bact. dysenteriae</i> ...	3	—	—	3	

1918. The cultures on agar plates gave pure smooth colonies which when transplanted to broth showed non-spontaneously agglutinated bacilli. A single colony was fished from an agar plate and transplanted successively for a period of twelve days in broth. At the end of the twelfth day it showed the presence of bacilli of the spontaneously and non-spontaneously agglutinating types.

Most of the colonies which developed after planting cultures freshly obtained from animals showed non-spontaneous-agglu-

tion. When these were sub-cultured at later periods, the colonies showed both agglutinating and non-agglutinating types.

In all instances the changes in the strain was from a pure to two types of colonies. The results of this work indicate that every member of the colon-typhoid group is changeable and may develop two types of colonies, dependent upon duration of cultivation or other circumstances of growth, as well as upon the type of artificial culture medium employed.

#### PHENOMENA OF SPONTANEOUS AGGLUTINATION IN VARIOUS FLUID CULTURE MEDIA (TABLE 4)

It has been observed, that spontaneous agglutination may occur in all ordinary fluid or solid culture media. Spontaneous agglutination occurs in a greater degree in glycerol broth, pepton water and all acid media, than it does in neutral or alkaline broth and it is more marked in concentrated than in dilute, liquid pepton medium. Cultures in alkaline broth usually remain clear and transparent, the presence of the alkali greatly retarding or completely inhibiting spontaneous agglutination.

When glucose broth was used as a culture medium, spontaneous agglutination of *Bact. typhosum* was prevented in many instances, but glucose broth cultures of *Bact. paratyphosum* A and B showed spontaneous agglutination in 24 hour cultures, and this has been even more marked with similar cultures of *Bact. coli*.

Growth in plain broth, either weakly acid or neutral in reaction, depends upon the type of colony. In some instances there is an excessive growth as well as a spontaneous agglutination. When such a culture was shaken or mixed with broth or salt solution, it disintegrated being thus differentiated from true spontaneous agglutination, in which disintegration occurs only when special methods are employed.

Plain broth or pepton water cultures which became spontaneously agglutinated were at first clear and after twenty-four hours a sediment was forming. If a cloudy growth is encountered in sub-culture, we are sure to find both spontaneous and non-spontaneous agglutination types of organisms present. This

TABLE 4  
*Spontaneous agglutination on various culture media*

SPONTANEOUS AGGLUTINATION OF CONTROL IN PLAIN BROTH	BACT. TYPHOSUM				BACT. PARATYPHOSUM A				BACT. PARATYPHOSUM B				BACT. COLI				
	-		+		-		+		-		+		-		+		
	No. 1	No. 2	No. 4	No. 5	No. 1	No. 2	No. 3	No. 4	No. 1	No. 2	No. 3	No. 4	No. 1	No. 2	No. 3	No. 4	Total
Number of strains .....	No. 1	No. 2	No. 4	No. 5	No. 1	No. 2	No. 3	No. 4	No. 1	No. 2	No. 3	No. 4	No. 1	No. 2	No. 3	No. 4	Total
Plain broth.....	-	-	-	-	1	2	1	7	2	1	2	7	2	1	2	7	8
One per cent glycerol broth.....	-	2	-	-	2	3	2	1	10	-	-	-	2	3	9	1	8
One per cent glucose broth.....	-	-	-	-	1	2	3	3	9	2	2	2	2	2	16	2	23
One per cent pepton water.....	-	2	-	-	2	3	2	1	10	1	1	1	3	2	3	14	11

Key: 3 strong; 2 medium; 1 weak agglutination; - Negative; + Spont. agglutinated.

can be determined by plating the culture and observing the two types of colonies. If flocculi, pellicles or small clumps are present in a twenty-four hour sub-culture of a non-spontaneously-agglutinating strain, we can assume that it has changed its characteristics and expect to find both types of growth present.

VARIATION IN GROWING POWER OF THE SPONTANEOUS AND NON-SPONTANEOUS AGGLUTINATION COLONIES FROM ONE STRAIN

Dreyer, using stock cultures for the agglutination test, passed a strain through several sub-cultures on broth medium and then treated a vigorously growing sub-culture with formalin. He reports that agglutination reactions with such cultures are more powerful.

Block (1897) claims that if too frequent transplantation of the culture is made there will occur in time spontaneous agglutination. This is possible only if an easily changeable strain containing spontaneous and non-spontaneous agglutination types is used. After isolating the non-agglutinating type of colony two broth cultures were made from a single colony, one of which was daily transplanted into fresh broth. After several days in the agar plate from the sub-cultures many colonies of spontaneously agglutinating types were present, while in the first broth culture which was not transplanted, there were only a few colonies of the spontaneously agglutinating types. Our deduction from these experiments is, that the type of organism showing spontaneous agglutination grows more vigorously than the non-spontaneously-agglutinating type taken from the strain in these experiments. On the other hand, if the growth of the non-spontaneously-agglutinating strain is stimulated, one will find that the spontaneously agglutinating type is weakened greatly and, in some cases, entirely disappears.

Experiments were made on growing the two types of culture together in broth, as follows: Broth cultures were prepared containing one loopful of a spontaneous and one, of a non-spontaneous agglutination type of organism. The results as shown on agar plates made at the end of each twenty-four hours varied widely. In some the growth of non-spontaneously agglutinating

bacilli daily exceeded that of spontaneously agglutinating bacilli; others showed a more vigorous growth of the latter type, and a few remained constant, showing parallel growth of the two types.

Other cultures which were not transplanted, showed a very slow change, while the daily transplanted culture showed in comparison very rapid changes in the proportion of the colony types. This varied directly with the original strains of the bacilli.

#### SPECIFIC AGGLUTINATING POWER BETWEEN TWO TYPES OF COLONIES OF ONE STRAIN

In experiments on specific agglutination with different types of colonies, no difference was found in the power of agglutination with two types of colonies of one strain.

Spontaneous agglutination differs chemically from specific serum agglutination, in that spontaneous agglutination can be prevented with most strains of *Bact. typhosum* and *Bact. paratyphosum* A and to a lesser extent with *Bact. paratyphosum* B, by adding 0.05 to 1 per cent formalin. Formalin in 0.05 to 0.2 per cent, does not interfere with specific serum agglutination, but slightly increases it if plain salt solution (0.85 per cent) has been used.

Many spontaneously agglutinating strains of *Bact. typhosum* when grown in glucose broth fail to show spontaneous agglutination. In others spontaneous agglutination is not inhibited by this means; neither does growth in glucose broth destroy the power of specific agglutination.

#### NOTES ON AGGLUTINATION TEST

In the agglutination test as applied to members of the colon-typhoid group it is necessary to differentiate between organisms giving spontaneous agglutination and organisms which do not agglutinate spontaneously. Organisms of the first group appear to give specific serum agglutination, but in the control bacillary suspension, when mixed with broth, pepton water,

salt solution or tap water (the diluents usually employed), they also give spontaneous agglutination.

Even old stock cultures which fail to give spontaneous agglutination at first, will do so, if the culture is inoculated into fresh media or repeatedly sub-cultured on agar, pepton water or broth. Spontaneous agglutination also appears in the suspension of a twenty-four-hour agar culture in water, salt solution, broth, pepton water or diluted serum.

If we examine an easily changeable stock culture, i. e., a culture containing both types of colonies, and streak such a culture on the agar plate, we will find the two types of colonies described above.

If upon plating we can isolate colonies of the non-spontaneously agglutinating type, only occasional sub-cultures from these colonies have to be made for the agglutination test, as such colonies invariably fail to agglutinate spontaneously, and further tests are unnecessary.

Agar cultures seem to show greater stability than fluid media cultures; some strains, however, always show one type of colony. For example certain strains of *Bact. typhosum* which have been handled for a year and a half showed constantly on both solid and fluid media non-spontaneous agglutination. Such a strain is valuable as a stock culture for the Widal test. In many of these cultures, even after being kept for several months in an ice chest, there was only one type of colony present corresponding with the original form, although some of the cultures were dead. These cultures were made on 0.5 per cent agar medium and the tubes were sealed with paraffin.

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# THE SOURCES AND CHARACTERISTICS OF THE BACTERIA IN DECOMPOSING SALMON

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In the course of an extensive investigation of the bacteriology of decomposing salmon a large number of cultures from various sources have been collected for study. The descriptions and, to some extent, the identification of the bacteria obtained from decomposing salmon caught on the spawning migration have been given in a previous report on this subject (Hunter, 1920). In that report the statement was made that the bacteria isolated from decomposing salmon were found to be those which are described in the literature as water, sewage and soil organisms. It was also stated that there was apparently no contamination of the fish with spore-forming organisms in the cannery. In order to determine definitely the relation between the flora of sea-water and that of decomposing salmon, experiments on the decomposition of "feedy" salmon, which have been described in a previous report (Hunter, 1921) were conducted at Astoria, Oregon. From the plates and the mixed cultures obtained from the decomposing salmon 197 cultures were selected for study.

Samples of sea-water were collected from various locations near the mouth of the Columbia River. From these water samples, plated on glucose agar, 14 cultures were selected for study. Although this number of cultures appears rather small,

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it may be taken as representative of the flora of the sea-water in that locality. Particular care was taken to avoid the selection of any more duplicates than was necessary and, since the plates from the water samples presented the same types of colonies repeatedly, the 14 cultures collected seemed fairly representative.

In order to extend this investigation of the bacterial flora of the salmon industry, a field laboratory was later established at Juneau, Alaska. Samples of sea-water were collected from various locations in southeastern Alaska. A large number of mixed cultures from different parts of the salmon canneries in this region were also collected to determine whether or not the bacterial flora of the cannery is identical with that of the sea-water and that of the decomposing salmon. From the water samples collected in Alaska 11 cultures were selected for further study. Here again the different types of colonies on the plates from the water samples were comparatively few and the 11 cultures obtained seemed representative of the bacterial flora of the sea-water in that region. From the mixed cultures obtained from the canneries 94 pure cultures were isolated and preserved for further study.

The organisms from sea-water and decomposing salmon and from the Alaskan canneries have been studied as separate groups and according to their morphology and their cultural reactions duplicates have been checked within each group. This has reduced the original number of 316 cultures to 85. The final designation of each of the 85 cultures with the number of original cultures included under this designation are given in table 1.

Each of the 85 cultures has been studied regardless of its action on salmon but particular attention has been given to the character which each organism may or may not possess of producing foul odors or indol in a specially prepared fish medium.<sup>2</sup>

<sup>2</sup> This medium was prepared in the following manner:

To 1000 grams of finely chopped saltwater trout, or weakfish, from which the skin and bones had been removed, was added 1000 cc. of distilled water and 15 grams of pepton. The infusion was made by heating in the Arnold sterilizer or on a water bath at 95° to 100°C. for one hour with occasional stirrings. The juice was strained through cheese cloth with a meat press, filtered through cotton and the reaction adjusted to neutral. The infusion was then heated in the

TABLE I  
*Summary of the number of cultures studied*

CULTURE	NUMBER OF ORIGINAL CULTURES INCLUDED UNDER THIS DESIGNATION	CULTURE	NUMBER OF ORIGINAL CULTURES INCLUDED UNDER THIS DESIGNATION	CULTURE	NUMBER OF ORIGINAL CULTURES INCLUDED UNDER THIS DESIGNATION
W <sub>1</sub>	2	360	11	443	2
W <sub>1a</sub>	1	366	1	444	3
W <sub>2</sub>	4	374	12	451	1
W <sub>3</sub>	3	380	12	452	2
W <sub>3a</sub>	1	390	7	452 <sub>a</sub>	1
W <sub>3b</sub>	1	395	1	453 <sub>a</sub>	1
W <sub>6</sub>	1	397	1	454	3
		399	1	456	9
W <sub>6a</sub>	1	400	1	458	2
W <sub>7a</sub>	2	401	1	459 <sub>a</sub>	1
W <sub>7b</sub>	1	403	1	460	7
W <sub>7c</sub>	1	406 <sub>a</sub>	2	461	1
W <sub>9b</sub>	1	410	3	462	2
W <sub>12</sub>	2	416	4	463	3
		417	1	463 <sub>a</sub>	1
W <sub>12a</sub>	1	419	1	464	3
W <sub>12</sub>	3	420 <sub>c</sub>	1	466	2
		420 <sub>E</sub>	1	467	1
H <sub>2</sub>	1	421	1	470 <sub>a</sub>	1
I	1	424	3	471 <sub>a</sub>	1
301 <sub>a</sub>	3	425	4		
303	4	427	2	473	2
314 <sub>a</sub>	3	429	1	480	1
316 <sub>b</sub>	1	429 <sub>a</sub>	1	503	6
323	5	430	8	510	7
325	52	431 <sub>a</sub>	1	513 <sub>a</sub>	1
343	3	432	1	516	30
351 <sub>a</sub>	1	436 <sub>a</sub>	2	518	17
354	1	439	4	523	5
		440	5	525	5

Each organism was grown in this medium for one week at 30°C. and the presence or absence of abnormal odors noted. The morphology and cultural reactions of 79 of the 85 cultures are

Arnold for thirty minutes at 100°C. and filtered, using folded filter papers. About 10 cc. was placed in each tube with about 1.5 grams of raw fish. For anaerobic cultures the surface was covered with a layer of liquid petrolatum. The medium was sterilized in the autoclave for fifteen minutes at 15 pounds.

given in tables 2, 3, 4, 5, 6, 7, 8 and 9. For convenience in presentation, the cultures from sea-water, salmon and the Alaskan canneries are given separately. The cultures from sea-water are given in tables 2 and 3 according to their ability to liquefy gelatin and regardless of pigment production. The cultures from decomposing salmon are described in tables 4, 5 and 6. Table 4 contains the gelatin-liquefying bacteria exclusive of pigment producers, table 5 the gelatin non-liquefying bacteria exclusive of pigment producers and table 6 the pigment producing bacteria. The cultures from the Alaskan canneries are described in tables 7, 8 and 9, and are divided according to gelatin liquefaction and pigment production in the same manner as those presented in tables 4, 5 and 6.

In recording the odor produced by the individual bacteria in fish broth three terms are used. The term "normal" signifies that no abnormal odor was produced and that the organism alone has no physically discernible effect on the fish. The term "off" is used to describe any abnormal odor which is not putrid or exactly foul but indicates that decomposition is taking place. The use of the term "foul" is obvious. While some of the organisms alone had no apparent effect on the fish, it was possible that these organisms, when grown in fish broth in mixed culture with other organisms might aid in the decomposition. With this in mind several combinations of bacteria were inoculated into fish broth in flasks and incubated for one week at 30°C. All the cultures from sea-water were inoculated into one flask and in one week produced a distinctly putrid odor. Four different combinations of bacteria, the sources of which were decomposing salmon, produced an extremely putrid, foul odor in the fish broth. A mixed culture of all the organisms isolated from the canneries produced a foul, but not a distinctly putrid, odor in this medium. The odor produced by the organisms growing in combination was in each case much worse than when grown individually.

Six cultures which have been carried along as individual cultures are not included in tables 2 to 9. These are 351<sub>a</sub>, a pink yeast; 451, 459<sub>a</sub>, 470<sub>a</sub> and 480, white yeasts; and 366, a culture

of Actinomyces. Culture 351<sub>a</sub> was isolated from the flesh of the back of a decomposing salmon and 366 was obtained from the intestines of a salmon. Cultures 451, 459<sub>a</sub> and 480 were isolated from canneries in Ketchikan, Alaska, and 470<sub>a</sub> was obtained from the cannery at Yes Bay, Alaska. Of the 79 cultures described in tables 2 to 9, 72 are rod-shaped organisms without spores, 3 are spore-forming rods and 4 are streptococci.

In studying these bacteria but little attempt has been made to identify as specific organisms any except the lactose fermenting organisms and the fluorescent bacteria. The attempt has been rather to determine what relation, if any, exists between the sea-water flora and the flora from decomposing salmon and from the canneries. The extensive work done by several groups of investigators, notably Winslow, Kligler and Rothberg (1919) and Levine (1918), make it possible to identify members of the colon-aerogenes group and, as far as possible, the lactose fermenting bacteria in this collection have been identified. The descriptions of fluorescent bacteria given by Edson and Carpenter (1912) and by Tanner (1918) also make it possible to identify bacteria of this group. The inadequate descriptions of non-fermenting, asporogenous, gelatin liquefying and non-liquefying bacteria given in the literature, if they are given at all, make it very difficult to identify organisms of this kind.

Examination of table 2 shows that of the 9 gelatin liquefying cultures from sea-water 1 is *Bact. cloacae* and 2 are *Ps. fluorescens*. W<sub>3</sub> differs from W<sub>2</sub> only in its failure to reduce nitrates. Two cultures in this table are unpigmented forms fermenting none of the sugars; one of the cultures, W<sub>13</sub>, produces spores. Two cultures are pigmented organisms which show no fermentative reactions. One culture, W<sub>13</sub>, produces spores and ferments glucose and sucrose with the production of acid but no gas and one culture, W<sub>7c</sub>, ferments glucose, lactose and sucrose with the production of acid.

In table 3 one culture, W<sub>12</sub>, was identified as *Bact. aerogenes*. One culture produces an acid fermentation in glucose; two cultures are unpigmented bacteria which do not ferment and two cultures produce yellow pigment and show no fermentative reactions.

TABLE 2  
*Gelatin liquefying bacteria from sea-water*

CUL- TURE	SOURCE	SHAPE	GRAM STAIN	LITMUS MILK	BIOCHEMICAL REACTIONS							FISH BROTH	PIGMENT	TYPE RESEMBLED
					Indol	Nitrate reduced	Glucose	Lactose	Sucrose	Methyl red	Voges-Proskauer			
W <sub>1a</sub>	Water from Il-waco, Wash.	Small rods	0	Peptonized	+	0	0	0	0	0	0	0	White	
W <sub>3a</sub>	Water from Il-waco, Wash.	Medium size rods	0	Peptonized	0	+	0	0	0	0	0	0	Flesh color	
W <sub>13</sub>	Water from Il-waco, Wash.; Haines, Alaska; Lynn Canal, Alaska	Large rods with spores	0	Peptonized	0	+	0	0	0	0	0	0	White	
W <sub>9b</sub>	Water from Lynn Canal, Alaska	Small rods	+	Decolorized	0	+	0	0	0	0	0	0	Orange	
W <sub>2</sub>	Water from Il-waco, Wash., 4 miles off Oregon Coast; Lynn Canal, Alaska	Medium size rods	0	Peptonized	+	A*	0	0	0	0	0	0	Fluorescent	<i>Ps. fluorescens</i>

W <sub>s</sub>	Water from Il- waco, Wash.; 4 miles off Oregon Coast; Tee Harbor, Alaska	Medium size rods	0	Peptonized	0	+	A	0	0	0	0	0	Off	Fluores- cent	<i>Ps. fluorescens</i>
W <sub>1</sub>	Water from Il- waco, Wash., and 4 miles off Oregon Coast	Large rods and spores	0	Peptonized	0	+	A	0	A	0	0	0	Normal	White	
W <sub>7c</sub>	Water from Haines, Alas- ka	Medium size rods	0	No change	+	+	A	A	A	0	0	0	Normal	White	
W <sub>6</sub>	Water from Chinook, Wash.	Medium size rods	0	Coagulated, peptonized	+	+	AG†	AG	AG	0	0	0	Foul	White	<i>Bact. cloacae</i>

\* A indicates acid but no gas.

† AG indicates acid and gas.

TABLE 3  
*Gelatin non-liquefying bacteria from sea-water*

CULTURE	SOURCE	SHAPE	GRAM STAIN	LITMUS MILK	BIOCHEMICAL REACTIONS							FISH BROTH PIGMENT	TYPE RESEMBLED	
					Inol	Nitrate reduced	Glucose	Lactose	Sucrose	Methyl red	Voges-Proskauer			
W <sub>3B</sub>	Water from H-waco, Wash.	Medium size rods	+	Decolorized	0	+	0	0	0	0	0	Normal	Yellow	
W <sub>6a</sub>	Water from Chinook, Wash.	Small rods	+	No change	0	+	0	0	0	0	0	Normal	Yellow	
W <sub>7B</sub>	Water from Haines, Alaska	Small rods	0	Decolorized	0	+	0	0	0	0	0	Off	White	
W <sub>12a</sub>	Water from Funtler Bay, Alaska	Short, thick rods	0	No change	+	+	0	0	0	0	0	Normal	White	
W <sub>7a</sub>	Water from Haines, Alaska, and Tee Harbor, Alaska	Small rods	0	No change	0	+	A*	0	0	0	0	Normal	White	
W <sub>12</sub>	Water from Tee Harbor, Alaska, and Funtler Bay, Alaska	Medium size rods	0	Acid coagulated	0	+	AG†	AG	AG	0	+	Off	White	<i>Bact. aerogenes</i>

 \* A indicates acid<sup>†</sup> but no gas.

† AG indicates acid and gas.



Of the 10 cultures described in table 4, 3 were identified as *Bact. cloacae* although in the case of 323 and 380 the methyl-red and the Voges-Proskauer tests are not typical. Repeated plating and testing of these two cultures did not alter the results of these tests and 323 and 380 have necessarily been recorded as atypical strains. Culture 316B cannot be confirmed as *Bact. cloacae* since it does not ferment sucrose. In all other reactions it is similar to *Bact. cloacae*. One culture, 397, in table 4 is a spore-forming organism which does not ferment any of the sugars; one culture, 395, ferments glucose with the production of acid but no gas; one culture, 354, produces acid in glucose and sucrose and three cultures, 399, 400 and 513<sub>a</sub>, produce acid in glucose, lactose and sucrose. Culture 513<sub>a</sub> is a streptococcus.

In table 5 one culture, 525, was identified as *Bact. communior*, one, H<sub>2</sub>, as *Bact. aerogenes* and two, 343 and 523, as *Bact. coli*, although 343 is atypical in that it gives a positive result in both the methyl-red and the Voges-Proskauer reactions. Culture 503 produces acid and gas in glucose and gives a positive methyl-red test. Three cultures are streptococci, two of which, 516 and 518, produce acid in glucose, lactose and sucrose and one of which, 510, produces acid only in glucose and lactose. One culture, 406, produces an acid fermentation in glucose. The two remaining cultures in this table, 314<sub>a</sub> and 410, show no fermentative reactions.

Among the pigment producing bacteria described in table 6 are two strains of *Ps. fluorescens*, 325 and 374. Culture 374 differs from 325 only in its failure to produce indol. Four of the cultures in this table show no fermentative reactions. Culture 401 produces acid in glucose and culture 403 has an acid fermentation in glucose, lactose and sucrose.

Of the 11 cultures from canneries described in table 7, two, 417 and 419, were identified as *Bact. cloacae*, although 419 must be recorded as atypical inasmuch as it gives negative results with both the methyl-red and the Voges-Proskauer reactions. Two cultures, 471<sub>a</sub> and 473, produce acid in glucose, lactose and sucrose and one culture, 429, produces an acid fermentation in glucose only. The remaining six cultures in this table show no fermentative reactions.

TABLE 4  
Gelatin liquefying bacteria from salmon exclusive of pigment producers

CUL- TURE	SOURCE	SHAPE	GRAM STAIN	LITMUS MILK	BIOCHEMICAL REACTIONS							FISH BROTH TYPE RESEMBLED	
					Indol	Nitrate reduced	Glucose	Lactose	Sucrose	Methyl red	Voges-Proskauer		
397	Stomach	Largerods with spores	0	Peptonized	0	+	0	0	0	0	0	0	Normal
395	Heart	Large rods	0	Coagulated	0	+	A*	0	0	0	0	0	Off
354	Intestines	Small rods	0	Peptonized	+	+	A	0	A	0	0	0	Off
399	Blood	Medium size rods	0	Decolorized	+	+	A	A	A	0	0	0	Off
400	Mouth	Small rods	0	Acid-coagulated	0	0	A	A	A	0	0	0	Off
513 <sub>a</sub>	Back	Streptococci	+	No change	0	+	A	A	A	+	0	0	Normal
316 <sub>b</sub>	Belly	Small rods	0	Coagulated, pep- tonized	+	+	AG†	AG	0	0	+	+	Very foul
323	Pew wound, belly, stomach	Medium size rods	0	Acid-coagulated, peptonized	+	+	AG	AG	AG	+	+	+	Foul <i>Bact. cloacae</i>
380	Back, belly, pew wound, mouth, intestines, heart, liver	Short, thick rods	0	Acid-coagulated, peptonized	0	+	AG	AG	AG	+	0	0	Off <i>Bact. cloacae</i>
390	Belly, pew wound, intes- tines, kidney	Medium size rods	0	Acid-coagulated, peptonized	0	+	AG	AG	AG	0	+	+	Foul <i>Bact. cloacae</i>

\* A indicates acid but no gas.

† AG indicates acid and gas.

TABLE 5  
Gelatin non-liquefying bacteria from salmon exclusive of pigment producers

CUL- TURE	SOURCE	SHAPE	GRAM STAIN	LITMUS MILK	BIOCHEMICAL REACTIONS							FISH BROTH	TYPE RESEMBLED	
					Indol	Nitrate reduced	Glucose	Lactose	Sucrose	Methyl red	Voges-Proskauer			
314 <sub>a</sub>	Back, belly, intestines	Small rods	0	No change	0	+	0	0	0	0	0	0	Normal	
410	Pew wound, blood, gills	Short thick rods	0	No change	0	0	0	0	0	0	0	0	Normal	
406	Mouth, belly, intestines	Short thick rods	0	No change	0	+	A*	0	0	0	0	0	Normal	
510	Back, belly, stomach	Streptococci	+	Decolorized	0	0	A	0	0	0	0	0	Off	
516	Back, belly, pew wound, intestines, stomach, kidney	Streptococci	+	Acid	+	0	A	A	A	+	0	0	Normal	
518	Back, belly, pew wound, intestines	Streptococci	+	Acid	0	0	A	A	A	+	0	0	Normal	
503	Back, belly	Medium size rods	0	Acid	0	+	AG†	0	0	+	0	0	Foul	
343	Pew wound, back, stomach	Small rods	0	Acid	+	+	AG	AG	0	+	+	+	Off	<i>Bact. coli</i>
523	Back, pew wound, stomach	Medium size rods	0	Acid	+	+	AG	AG	0	+	0	0	Foul	<i>Bact. coli</i>
H <sub>2</sub>	Pew wound	Medium size rods	0	Acid	0	+	AG	AG	AG	0	+	+	Normal	<i>Bact. aerogenes</i>
525	Belly, pew wound, intestines	Medium size rods	0	Acid	+	+	AG	AG	AG	+	+	0	Normal	<i>Bact. communior</i>

\* A indicates acid but no gas.

† AG indicates acid and gas.

One culture, 424, in table 8 was identified as *Bact. aerogenes*; one culture, 464, produces acid in glucose and sucrose and two cultures, 454 and 463, produce acid in glucose only. The other eight cultures described in this table do not ferment any of the sugars.

Among the twelve cultures in table 9 are two, 431<sub>a</sub> and 467, which produce acid in glucose, lactose and sucrose; one, 461, which produces acid in glucose and lactose and one, 443, which produces acid in glucose only. The remaining 8 cultures show no fermentative reactions.

Throughout the investigation no obligate anaerobes were isolated. Anaerobic cultures were made from the material investigated and several cultures isolated which were at first regarded as anaerobic bacteria but subsequent work showed them to be facultative. This was especially true of the streptococci isolated from salmon. These streptococci were originally isolated from anaerobic cultures and they appear to grow best under anaerobic conditions. They do grow fairly well on agar slants, however, and have been carried along in this work as aerobic organisms.

As a result of this study of the morphology and the cultural reactions of these 79 organisms it has been possible in several instances to identify as the same organism cultures from the various sources and in this way to establish a partial correlation between the bacterial floras of sea-water, of decomposing salmon and of the Alaskan canneries. This has been done where the morphology and cultural reactions are such that there can be no doubt that the cultures are identical even though it were impossible to identify them as to group or species. This correlation is shown in table 10.

Cultures W<sub>2</sub> and 325 are both the same strain of *Ps. fluorescens*. Reference to table 1 will show the relative abundance of this organism among the original 316 cultures. This organism appears to be widely distributed in the sea-water since it was obtained from samples collected at Ilwaco, Wash., four miles off the Oregon Coast and Lynn Canal, Alaska (see map, fig. 1). It was also obtained 52 times from the various parts of

TABLE 6  
Pigment producing bacteria from salmon

CUL- TURE	SOURCE	SHAPE	PIGMENT	GRAM STAIN	GELATIN LIQEFIED	LITMUS MILK	BIOCHEMICAL REACTIONS						FISH BROTH	TYPE RESEMBLED		
							Indol	Nitrate reduced	Glucose	Lactose	Nucrose	Methyl red			Voges-Proskauer	
I 301 <sub>a</sub>	Pew wound Back, pew wound	Small rods	Orange	0	0	Decolorized	0	+	0	0	0	0	0	0	Normal	
303	Back, belly	Small rods	Yellow	+	0	No change	0	+	0	0	0	0	0	0	Normal	
360	Back, belly, pew wound, stomach	Small rods	Lenon yellow	+	0	Decolorized	0	+	0	0	0	0	0	0	Normal	
325	Back, belly, pew wound, stomach, in- testines, gills, mouth, liver, kidney	Small rods	Flesh color	0	+	Peptonized	+	+	0	0	0	0	0	0	Foul	
374	Back, belly, pew wound, stomach, in- testines, gills, mouth, liver, kidney	Medium size rods	Fluores- cent	0	+	Peptonized	+	+	A*	0	0	0	0	0	Off	<i>Ps. fluores- cens</i>
401	Back, pew wound, stom- ach, intes- tines, heart, liver, blood	Medium size rods	Fluores- cent	0	+	Peptonized	0	+	A	0	0	0	0	0	Off	<i>Ps. fluores- cens</i>
403	Gills	Medium size rods	Orange	0	+	Peptonized	0	+	A	0	0	0	0	0	Foul	
403	Liver	Small rods	Yellow	0	0	Decolorized	+	+	A	A	A	A	0	0	Off	

\* A indicates acid but no gas.

TABLE 7  
*Gelatin liquefying bacteria from Alaska salmon canneries exclusive of pigment producers*

CUL- TURE	SOURCE	SHAPE	GRAM STAIN	LITMUS MILK	BIOCHEMICAL REACTIONS							FISH BROTH TYPE RESEMBLED		
					Indol	Nitrate reduced	Glucose	Lactose	Sucrose	Methyl red	Voges-Proskauer			
416	Douglas Ketchikan	Medium size rods	0	Decolorized	0	+	0	0	0	0	0	0	Normal	
420 <sub>c</sub>	Haines	Small rods	0	Acid	0	+	0	0	0	0	0	0	Foul	
427	Ketchikan	Short, thick rods	0	Acid	0	0	0	0	0	0	0	0	Normal	
429 <sub>a</sub>	Excursion Inlet Excursion Inlet	Medium size rods	0	Decolorized	0	+	0	0	0	0	0	0	Foul	
432	Hoonah	Slender rods	+	Peptonized	0	+	0	0	0	0	0	0	Normal	
460	Sitka, Hawk Inlet, Ket- chikan, Hoonah, Met- lakatla	Small rods	+	Acid	0	+	0	0	0	0	0	0	Off	
429	Excursion Inlet	Short, thick rods	+	No change	+	+	A*	0	0	0	0	0	Normal	
471 <sub>a</sub>	Yes Bay	Small rods	0	Acid-coagu- lated	0	0	A	A	A	0	0	0	Off	
473	Yes Bay, Ketchikan	Medium size rods	0	Coagulated, peptonized	0	+	A	A	A	0	0	0	Normal	
417	Haines	Medium size rods	0	Coagulated, peptonized	0	+	AG†	AG	AG	0	+	+	Foul	<i>Bact. cloacae</i>
419	Haines	Medium size rods	0	Acid, pepton- ized	+	+	AG	AG	AG	0	0	0	Foul	<i>Bact. cloacae</i>

\* A indicates acid but no gas.

† AG indicates acid and gas.

TABLE 8  
*Gelatin non-liquefying bacteria from Alaskan salmon canneries exclusive of pigment producers*

CUL- TURE	SOURCE	SHAPE	GRAM STAIN	LITMUS MILK	BIOCHEMICAL REACTIONS							FISH BROTH TYPE RESEMBLED		
					Indol	Nitrate re- duced	Glucose	Lactose	Sucrose	Methyl red	Voges Proskauer			
430	Excursion Inlet, Hoonah, Sitka, Ketchikan, Metlakatla	Small rods	0	Decolorized	0	+	0	0	0	0	0	0	0	Normal
436 <sub>a</sub>	Hawk Inlet, Chomley	Medium size rods	0	Decolorized	0	+	0	0	0	0	0	0	0	Off
439	Sitka	Small rods	0	Acid	0	+	0	0	0	0	0	0	0	Normal
440	Sitka, Chomley, Metlakatla, Ketchikan	Medium size rods	0	Decolorized	0	0	0	0	0	0	0	0	0	Off
452	Sitka, Ketchikan	Small rods	+	Acid	0	0	0	0	0	0	0	0	0	Normal
452 <sub>a</sub>	Sitka	Medium size rods	0	No change	+	0	0	0	0	0	0	0	0	Off
453 <sub>a</sub>	Ketchikan	Small rods	0	Decolorized	+	+	0	0	0	0	0	0	0	Off
458	Ketchikan, Hawk Inlet	Small rods	0	Decolorized	+	+	0	0	0	0	0	0	0	Off
454	Ketchikan	Short, thick rods	0	No change	0	+	A*	0	0	0	0	0	0	Normal
463	Chomley, Yes Bay, Metlakatla	Short, thick rods	0	Decolorized	+	+	A	0	0	0	0	0	0	Normal
464	Chomley, Yes Bay	Slender rods	0	Acid	0	0	A	0	A	0	+	0	0	Normal
424	Haines, Tee Harbor	Medium size rods	0	Acid	0	+	AG†	AG	AG	AG	0	+	+	Foul <i>Bact. aerogenes</i>

\* A indicates acid but no gas.

† AG indicates acid and gas.

TABLE 9  
*Pigment producing bacteria from Alaskan salmon canneries*

CUL- TURE	SOURCE	SHAPE	PIGMENT	GRAM STAIN	GELATIN LIQ- UID	LITMUS MILK	BIOCHEMICAL REACTIONS						FISH BROTH	
							Indol	Nitrate Re- duced	Glucose	Lactose	Sucrose	Methyl red		Voges Proskauer
420 <sub>E</sub>	Haines	Small rods	Orange	0	0	Decolorized	0	+	0	0	0	0	0	Normal
421	Tee Harbor	Small rods	Flesh color	0	+	Peptonized	+	+	0	0	0	0	0	Foul
425	Hoonah, Chatham, Metlakatla, Excursion Inlet	Medium size rods	Orange	0	+	Decolorized	+	+	0	0	0	0	0	Normal
444	Hawk Inlet, Chat- ham, Sitka	Small rods	Lemon yel- low	+	+	Slightly alkali- line	0	+	0	0	0	0	0	Off
456	Hoonah, Chatham, Juneau, Excursion Inlet, Chomley, Ketchikan, Yes Bay	Medium size rods	Lemon yel- low	+	0	No change	0	+	0	0	0	0	0	Foul
462	Chomley, Metlakatla	Small rods	Flesh color	0	+	Decolorized	+	+	0	0	0	0	0	Normal
463 <sub>A</sub>	Chomley	Short, thick rods	Yellow	0	0	Decolorized	0	+	0	0	0	0	0	Normal
466	Metlakatla, Ketchi- kan	Medium size rods	Lemon yel- low	0	0	No change	0	0	0	0	0	0	0	Normal
443	Sitka, Chatham	Medium size rods	Orange	0	+	Peptonized	0	+	A*	0	0	0	0	Foul
461	Chomley	Medium size rods	Yellow	0	0	Decolorized	0	+	A	A	0	0	0	Normal
431 <sub>A</sub>	Hoonah	Short, thick rods	Lemon yel- low	+	+	Coagulated	0	+	A	A	A	+	0	Normal
467	Metlakatla	Small rods	Yellow	0	0	Decolorized	+	+	A	A	A	0	0	Normal

\* A indicates acid but no gas.



the salmon as described in table 10. Cultures  $W_3$  and 374 are also identical strains of *Ps. fluorescens* isolated from sea-water in widely separated localities and from the various parts of decomposing salmon. As shown in table 1 this organism was obtained from water 3 times and from salmon 12 times. *Ps. fluorescens* was not found among the 94 cultures from Alaskan canneries.

Cultures  $W_{3a}$  and 301<sub>a</sub> are yellow bacteria identical in their morphology and cultural reactions. This organism was obtained once from sea-water collected at Ilwaco, Wash., and three times from salmon. Cultures  $W_{6a}$ , 303 and 456 are also yellow pigment producing bacteria with identical morphology and cultural reactions isolated once from sea-water, four times from salmon and nine times from Alaskan canneries. References to table 10 and to the map (fig. 1) will show that this organism was found in widely separated areas throughout Southeastern Alaska. A third yellow organism, represented in the tables as 403 and 467, was obtained once from salmon and once from a cannery at Metlakatla.

Cultures  $W_6$ , 390 and 417 were identified as a strain of *Bact. cloacae* isolated once from water collected at Chinook, Wash., seven times from salmon and once from a cannery at Haines, Alaska (see map, fig. 1). Since 316<sub>b</sub>, 323, 380 and 419 each represent an atypical strain of *Bact. cloacae* they have not been considered as identical with  $W_6$ , 390 and 417. It is possible, however, that the correlation might be extended to include the sources from which the atypical strains of this organism were isolated.

Cultures  $W_{7a}$ , 406 and 454 are identical and this organism was found twice in water from Alaska, twice from salmon and three times from canneries in Ketchikan, Alaska. It will be noted that Haines and Tee Harbor, Alaska, where this organism was found in the water are a great distance from Ketchikan (fig. 1) where it was found in the canneries. The fact that this organism was also isolated from salmon in Oregon indicates that it is widely distributed.

Cultures  $W_{7b}$  and 430 are bacteria having the same morphology and cultural reaction, isolated once from the water at Haines,

TABLE 10  
*Bacteria isolated from more than one source*

CULTURE	WATER	SALMON	ALASKAN CANNERY	TYPE
W <sub>2</sub> 325	Ilwaco, Wash. 4 miles off Oregon Coast, Lynn Canal, Alaska	Back, belly, pew wound, stomach, intestines, gills, mouth, liver, kidney		<i>Ps. fluorescens</i>
W <sub>3</sub> 374	Ilwaco, Wash. Tee Harbor, Alaska, 4 miles off Oregon Coast	Back, pew wound, stomach, intestines, heart, liver, blood		<i>Ps. fluorescens</i>
W <sub>3a</sub> 360 421	Ilwaco, Wash.	Back, belly, pew wound, stomach	Tee Harbor	
W <sub>3b</sub> 301 <sub>a</sub>	Ilwaco, Wash.	Back, pew wound		
W <sub>6</sub> 390 417	Chinook, Wash.	Belly, pew wound, intestines, kidney	Haines	<i>Bact. cloacae</i>
W <sub>6a</sub> 303 456	Chinook, Wash.	Back, belly	Hoonah, Juneau, Excursion Inlet, Chatham, Chomley, Ketchikan, Yes Bay	
W <sub>7a</sub> 406 454	Haines, Alaska Tee Harbor, Alaska	Mouth, belly	Ketchikan	

W <sub>7b</sub> 430	Haines, Alaska		Excursion Inlet, Hoonah, Sitka, Ketchikan, Metlakatla	
W <sub>7c</sub> 399 473	Haines, Alaska	Blood	Yes Bay, Ketchikan	
W <sub>12</sub> H <sub>2</sub> 424	Tee Harbor, Alaska Funter Bay, Alaska	Pew wound	Haines, Tee Harbor	<i>Bact. aerogenes</i>
W <sub>12a</sub> 314 <sub>a</sub> 458	Funter Bay, Alaska	Back, belly, intestines	Ketchikan, Hawk Inlet	
W <sub>13</sub> 397	Iliwaco, Wash. Haines, Alaska Lynn Canal, Alaska	Stomach		
I-120 <sub>c</sub> 400-471 <sub>a</sub> 401-443 403-467		Pew wound Mouth Gills Liver	Haines Yes Bay Sitka, Chatham Metlakatla	

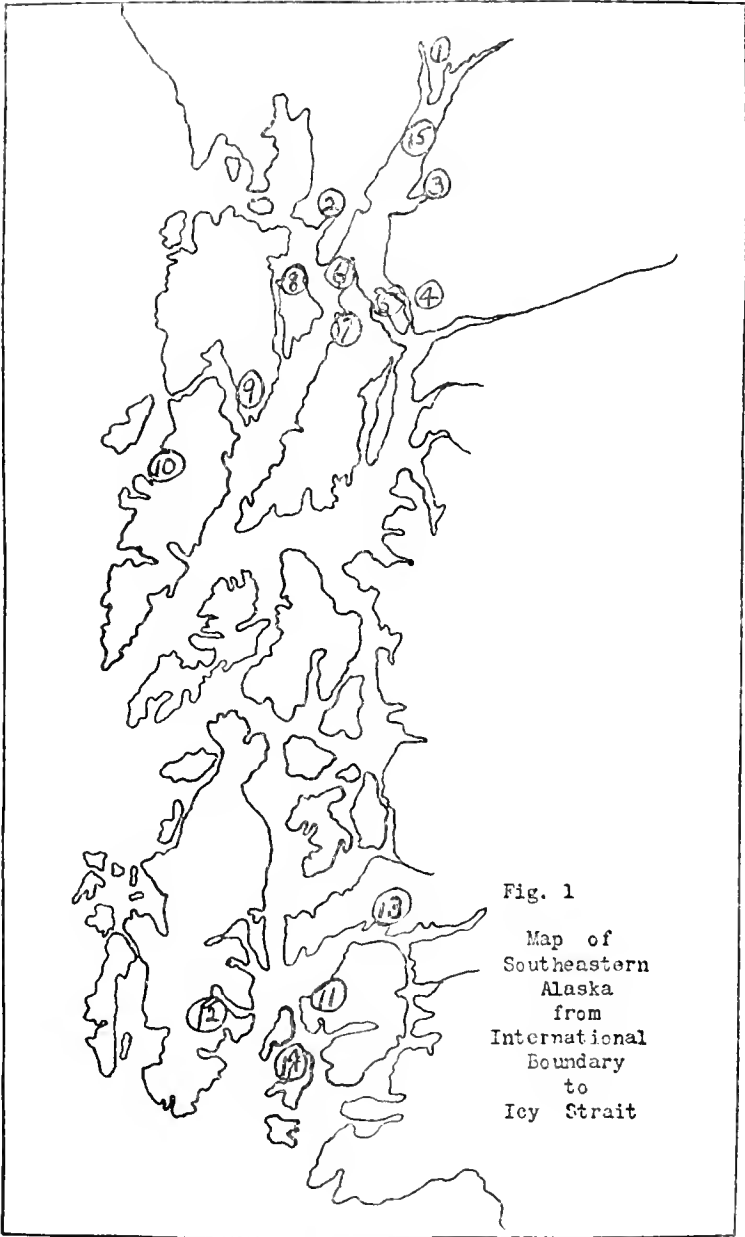


Fig. 1  
 Map of  
 Southeastern  
 Alaska  
 from  
 International  
 Boundary  
 to  
 Icy Strait

- |                    |               |                |
|--------------------|---------------|----------------|
| 1. Haines          | 6. Funter Bay | 11. Ketchikan  |
| 2. Excursion Inlet | 7. Hawk Inlet | 12. Chomley    |
| 3. Tee Harbor      | 8. Hoonah     | 13. Yes Bay    |
| 4. Juneau          | 9. Chatham    | 14. Metlakatla |
| 5. Douglas         | 10. Sitka     | 15. Lynn Canal |

Alaska, and eight times from canneries in Alaska extending from Excursion Inlet on the north to Metlakatla on the south. This culture was not found in decomposing salmon.

As seen in the tables, cultures W<sub>7c</sub>, 399 and 473 are identical in morphology and cultural reactions. This organism was isolated once from sea-water collected at Haines, Alaska, once from salmon and twice from canneries at Yes Bay and Ketchikan, Alaska.

Cultures W<sub>3a</sub>, 360 and 421 were identified as a flesh-colored organism isolated once from sea-water collected at Ilwaco, Wash., eleven times from salmon and once from a cannery at Tee Harbor, Alaska.

The culture of *Bact. aerogenes* isolated twice from sea-water from Alaska, once from salmon and three times from canneries at Haines and Tee Harbor, Alaska, is designated in the tables under W<sub>12</sub>, H<sub>2</sub> and 424.

Cultures W<sub>12a</sub>, 314<sub>a</sub> and 458 are identical. This organism was isolated once from sea-water from Funter Bay, Alaska, three times from salmon and twice from canneries at Ketchikan and Hawk Inlet. The isolation of this culture from material collected from such widely separated areas indicates that it is generally distributed throughout the whole region.

Only two spore-forming bacteria were found in the course of the work and one of these, represented in the tables as W<sub>13</sub> and 397 was isolated three times from sea-water collected at Ilwaco, Wash., Haines, Alaska, and Lynn Canal, Alaska. It was also isolated once from decomposing salmon. Although this organism appears to be widely distributed in the sea-water of that region it was not common in the decomposing salmon and was not found at all in the various parts of the canneries. When grown in pure culture this spore-former seems to have no effect on fish.

Cultures I and 420<sub>o</sub> are orange-colored organisms identical in morphology and cultural reactions isolated once from salmon and once from a cannery at Haines. It was not found in the water samples examined. An orange-colored organism, represented by 401 and 443, was isolated once from salmon and twice from canneries at Sitka and Chatham, Alaska.

Cultures 400 and 471<sub>a</sub> are identical and were isolated once from salmon and once from a cannery at Yes Bay, Alaska.

In considering the sources of these various cultures, it may be borne in mind that all the isolations from salmon were made in Oregon and the fact that some cultures isolated there are identical with those obtained in Alaska indicates that such organisms have a wide distribution.

The frequency with which some bacteria are found in the cultures from decomposing salmon, when considered in connection with their decomposing action on fish, indicates that these organisms play an important part in the decomposition of the salmon. As explained in a previous report (Hunter, 1921) the salmon cultures studied in this investigation were obtained on successive days from salmon which were held under known conditions. The predominance of *Ps. fluorescens* throughout the viscera and the muscular tissue of the decomposed salmon leads to the conclusion that this organism is an important factor in the decomposition of the salmon. Two other organisms appear to play an important part in the decomposition of the salmon, namely, *Bact. cloacae* and the flesh-colored organism designated as No. 360. These organisms appear repeatedly on the plates and cultures made from decomposing salmon and when grown in fish broth in pure culture they produce very foul odors. As stated before, the other bacteria present may be regarded as accessory in the decomposition of the salmon but from this investigation it seems evident that *Ps. fluorescens*, *Bact. cloacae* and the flesh-colored organism (360) are of greater importance than any of the others.

This investigation has not shown that there are any different bacteria introduced by the use of the pew, or single-tine fork, than are to be found in the sea-water or in the salmon as it comes from the sea-water.

In obtaining cultures from canneries particular attention was paid to the exact location within the cannery from which the material was collected. This was done to determine whether there was any chance of contamination of the canned fish with spore-formers before cooking and also to determine whether there was a bacterial flora peculiar to the cannery or whether

the bacteria found within the cannery were those which came from the salmon and the sea-water. The investigation has shown that many of the organisms collected are generally distributed throughout the cannery and are not restricted to any particular part. In the case of such cultures as 430, 456 and 460, which were found eight, nine and seven times respectively, the organisms were isolated all the way along the canning line from the butchering table to the retorts. Since the location within the cannery, from which the culture was obtained, has no particular significance in a report of this kind, no note of it has been made in the tables. It is thought sufficient to give simply the geographical location from which the culture was obtained.

Of the 79 cultures reported here only 39 or about 49 per cent were found to have a common source such as sea-water and salmon, sea-water and the cannery or salmon and the cannery. If a larger number of cultures had been collected from a large number of sources throughout the salmon canning region, it is very probable that this percentage of correlation would be increased. Of the 15 cultures from sea-water there are only three ( $W_1$ ,  $W_{1a}$ , and  $W_{9b}$ ) which were not also found in salmon, in the cannery or in both salmon and the cannery. Fifteen cultures from salmon are identical with cultures from other sources and 14 cultures were isolated from salmon and not isolated from sea-water or from the canneries. Of the 35 cultures reported from canneries only 12 were obtained from other sources. In considering the comparatively small number of cultures collected (316) over such a large area it is not surprising that not more than 49 per cent of them were isolated from the three sources. There are only four cultures in the collection from sea-water ( $W_1$ ,  $W_{1a}$ ,  $W_{7b}$  and  $W_{9b}$ ) which were not also found in decomposing salmon and it seems probable that if the number of cultures from salmon were larger it would include these four organisms. On the other hand it is also probable that, if it had been possible to collect a larger number of water samples and, hence, a larger number of cultures from water, very many of the 14 cultures from salmon, which it was impossible to correlate with any other source, would have been included in the sea-water flora. This is particularly true of such organisms as *Bact. coli*

and *Bact. communior*. It is apparent that, just as suggested previously, the bacteria causing decomposition in salmon are those forms the natural habitat of which is the sea-water from which the salmon are taken.

The correlation between the flora of the Alaskan canneries and the flora of the sea-water and the salmon is not as clear. Only about 34 per cent of the cultures from the canneries can be traced to another source and this leaves the source of 66 per cent of these cultures unexplained. There is the same probability existing here, however, that, if the number of sea-water cultures could have been increased the number of cannery organisms correlating with them might also have been increased. From this investigation, the outstanding fact about the bacterial flora of the Alaskan canneries is that it consists mainly of asporogenous, non-fermenting bacteria which appear to have very little effect on the decomposition of the salmon. This confirms the statement made in a previous report (Hunter, 1920) that the organisms concerned in the decomposition of salmon are those forms which are brought with the salmon from the sea-water and that the decomposition is not due to bacteria which contaminate the salmon within the cannery.

#### SUMMARY

In studying the distribution of the bacteria concerned in the decomposition of salmon, 316 cultures were collected from sea-water, from decomposing salmon and from salmon canneries throughout southeastern Alaska. By checking the duplicates this number was reduced to 85 cultures, one of which was an Actinomyces, one a pink yeast and four white yeasts. Of the remaining 79 cultures 72 were rod-shaped organisms without spores, 3 were spore-forming rods and 4 were streptococci. The morphology and cultural reactions of these 79 cultures are given.

While no attempt has been made to specifically identify many of the cultures, 6 have been identified as *Bact. cloacae*, 3 as *Bact. aerogenes*, 2 as *Bact. coli*, one as *Bact. communior* and 4 as *Ps. fluorescens*. The majority of the bacteria collected apparently belong to a large group of non-fermenting soil and water bacteria. These bacteria are similar to those mentioned by Jordan



(1903) in his report on the kinds of bacteria isolated from river water and are included in his Groups VIII to XIII inclusive. Conn (1917) stated that slowly liquefying or non-liquefying, non-spore-forming short rods such as these make up from 40 to 75 per cent of the organisms developing on aerobic plates inoculated with soil.

The results of this investigation indicate that *Ps. fluorescens*, *Bact. cloacae* and an unidentified flesh-colored organism play an important part in the decomposition of the salmon.

In determining the correlation between the bacteria from the three sources, it has been found that 80 per cent of the bacteria collected from sea-water are also found in decomposing salmon, in the canneries, or in both. Approximately 52 per cent of the salmon cultures were found elsewhere and about 34 per cent of the cultures from the Alaskan canneries were obtained from other sources.

The results of this investigation confirm the statement previously made (Hunter, 1920) that the bacteria concerned in the decomposition of salmon are those forms the natural habitat of which is the sea-water from which the salmon are taken and that the decomposition of salmon is not due to bacteria which contaminate the salmon within the cannery.

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# VIABILITY OF THE COLON-TYPHOID GROUP IN CARBONATED WATER AND CARBONATED BEVERAGES

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The destructive effect of carbon dioxide on various microorganisms and the value of carbonation for the preservation of foods and beverages have claimed the interest of a number of workers since the first days of bacteriology. As early as 1885, Leone reported the examination of several commercial mineral waters which were under a slight pressure of CO<sub>2</sub>. The number of microorganisms found to be present was always low. He also observed that after passing CO<sub>2</sub> gas through a drinking water the total count rapidly diminished.

Somewhat later than this a number of investigations were made of the destructive effect of CO<sub>2</sub> under relatively high pressures. Schaffer and Freudenreich (1891-1892), after studying the effect of pressures of 40 to 50 atmospheres of CO<sub>2</sub> combined with an increase of temperature, conclude that CO<sub>2</sub> has only a feeble bactericidal action. Sabrazès and Bazin (1893) found that cultures of *Bact. typhosum*, *Bact. coli*, *Staphylococcus aureus*, and the anthrax bacillus were able to develop after exposure to 60 to 70 atmospheres of CO<sub>2</sub> for several hours. These results are contradicted by D'Arsonval and Charrin (1893) who report that CO<sub>2</sub> under a pressure of 50 atmospheres sterilized cultures of *Ps. pyocyanea* in from six to twenty-four hours. Recently, Larson, Hartzell, and Diehl (1918), in a study of the effect of pressures upon bacteria, found that CO<sub>2</sub> under a pressure of 50 atmospheres would destroy *Bact. typhosum*, *Bact. coli*, *Mycobact. tuberculosis*, *Ps. pyocyanea*, staphylococci, strep-

toococi, and pneumococci, in a period of time ranging from one and one-half to two and one-half hours. Yeast cells were unaffected after an exposure of forty-eight hours.

Since these pressures are many times greater than those to which the ordinary carbonated beverages are subjected, there is the possibility that certain organisms may retain their vitality for a longer period. Several reports of the examination of carbonated beverages purchased in the open market have shown that occasionally there are encountered considerable numbers of microorganisms, including those indicative of pollution. Allen, LaBach, Pinnell, and Brown (1915) report a sanitary survey of the "soft drink" industry of Kentucky. Although carbonation was found to cause a distinct reduction in the numbers present, occasional high counts and the presence of *Bact. coli* were reported. Stokes (1920) recently examined a great variety of "soft drinks" and noted the frequent presence of *Bact. coli* in 10 cc. and 1 cc. quantities, with an occasional occurrence in 0.1 cc. The plate counts exhibited great variation, and while the majority of samples yielded counts of less than 100 per cubic centimeter, a few showed surprisingly high numbers. Gershenfeld (1920) reports similar results. Young and Sherwood (1911) have reported an experiment in which they determined the viability of *Bact. typhosum*, *Bact. coli*, and *Erythrob. prodigiosus* in carbonated water to which lemon syrup had been added. Although the typhoid bacillus showed a considerable reduction in numbers after four hours exposure, a few viable cells were found after ten days. *Bact. coli* and *Erythrob. prodigiosus* were found to be somewhat more resistant than *Bact. typhosum*.

In the present investigation chief emphasis has been placed upon the colon-typhoid group for the purpose of determining the length of time one may expect the various members of this group to withstand the environment of the different types of commercial carbonated beverages.

The following organisms have been employed: *Bact. coli* (fecal origin), *Bact. paratyphosum* B, and *Bact. typhosum*. Also, as a matter of interest, two common spore forms were included; *B. mesentericus*, and a putrefactive anaerobe of the *Clostridium sporogenes* group.

The beverages were prepared and carbonated in the 7-ounce bottles commonly used in the industry. Since they were prepared as nearly as possible under commercial conditions and no effort was made to sterilize the various ingredients, control examinations of the product were made previous to experimental inoculation to determine the absence of the particular type of organism used in the investigation. In no instance was any difficulty of this kind encountered. Throughout the work commercial CO<sub>2</sub> was used for carbonation. As a test for any impurities in the carbon dioxide which might affect the death-rate of the organisms, tap water was carbonated as usual, then heated in the Arnold sterilizer for a short period to expel the CO<sub>2</sub> and finally the death-rate of *Bact. coli* in this water was compared to that in parallel samples of the original tap water. No discrepancies other than those which might be attributed to experimental variation were observed.

Small amounts of a suspension of the various test organisms in sterile tap water were used for inoculation. This was accomplished in one of the two following ways. The first method consisted of adding equal amounts of bacterial suspension to each bottle just before carbonation. In the second method the samples were prepared, bottled, carbonated, and capped as usual. They were then stored at 1°C. for several days until used, when the bottles were re-opened and inoculated. If opened while still cool, there was little loss of CO<sub>2</sub> gas. The first method was used for most of the experiments with *Bact. coli*. The second method was necessary when working with *Bact. typhosum* and *Bact. paratyphosum* B since by the first method there is more or less spattering of the material during the process of carbonation.

Immediately after inoculation and at definite intervals thereafter plate counts were made. To prevent the considerable loss of CO<sub>2</sub> upon repeated opening of the same bottles, especially those held at room temperature, a number of bottles were inoculated with equal amounts of bacterial suspension and, at each time interval, different sets of two were opened and samples withdrawn for plating. When the numbers had become so

reduced as to give negative results upon plating 1 cc. quantities, larger amounts, 5 cc. and 10 cc., were introduced into broth to determine, insofar as possible, the final disappearance of the organisms in question. This was done by streaking Endo plates from the broth cultures, fishing any typical colonies, and finally applying the usual methods used for the identification of the various members of this group of organisms.

#### EXPERIMENTAL

Since temperature may be expected to exert a marked influence upon the death-rate, experimental samples were held at two different temperatures, namely, in cold storage at 1°C. and at room temperature, 19° to 23°C. Tables 1 and 2 present data showing the viability of *Bact. coli* in carbonated water at several different pressures and also, for purposes of comparison, in plain tap water. It is evident that carbonation causes a speedy destruction of the colon bacillus and that this effect is dependent upon the temperature at which the samples are held, being much more pronounced at room temperature than at 1°C. Furthermore, the different degrees of pressure of CO<sub>2</sub> mentioned in these tables apparently exerted little or no influence upon viability, for the organisms were killed as speedily in water saturated with CO<sub>2</sub> (at both 20°C. and 1°C.), but under no excess pressure, as they were in the carbonated samples under pressures of 28 and 41 pounds per square inch. In fact, where the pressure was released the plate counts frequently were less than those of the samples held under pressure (table 2), a phenomenon which was regularly observed upon several repetitions of the experiment.

To gain an idea of the hydrogen-ion concentration of carbonated water the indicators brom-phenol-blue and methyl-red were added to different bottles which were then filled with carbonated water at these several pressures. In this way the value was roughly determined as pH 4.0-4.4. Release of the pressure, as indicated in table 2, was followed by very little, if any, immediate change in the hydrogen-ion concentration when meas-

ured in this way. When, however, such samples are held for a period of one or two weeks at 19° to 20°C. there is a gradual escape of CO<sub>2</sub> gas as evidenced by a decrease in the hydrogen-ion concentration. It is believed that under the conditions of our experiments the acidity of the dissociated carbonic acid is

TABLE I

Showing the comparative viability of *Bact. coli* in carbonated tap water under pressure and in plain (non-carbonated) tap water

	TIME INTERVAL	CARBONATED TAP WATER PRESSURE 41 POUNDS PER SQUARE INCH (2.78 ATMOSPHERES) AT 18°C.		CONTROLS, PLAIN TAP WATER	
Held at room temperature (20-21°C.)	At once	181,000*	156,000	201,000	190,000
	4 hours	79,000	80,000		
	24 hours	950	6,600	203,000	191,000
	4 days	1 cc. plate negative	26	34,200	18,000
	7 days	10 cc. 0†	10 cc. +	14,800	10,000
		5 cc. +	5 cc. 0		
	11 days	1 cc. 0			
Held at 1°C.	At once	163,000	181,000	200,000	260,000
	24 hours	51,000			
	4 days	25,500	23,000	166,000	180,000
	7 days	2,700	5,400	190,000	Lost
	14 days	1 cc. plate negative	30	100,000	Lost
	26 days	5 cc. +	1 cc. +	17,000	Lost
		1 cc. 0	0.1 cc. +		

\* Figures represent numbers of *Bact. coli* per centimeter.

† 0 indicates the absence, and + the presence, of *Bact. coli* as determined by transferring the specified amount of water (10, 5 or 1 cc.) to broth. This was done when the numbers had become so reduced as to give negative results upon plating 1 cc. quantities.

the main factor responsible for the death of the bacteria. Other factors, such as differences in osmotic pressure, may also play a part.

Several of the simpler carbonated beverages were used in the next experiments. It should be realized that certain acids—

usually citric, tartaric, phosphoric, or lactic—are added to some types of beverages and that these acids may affect the longevity of the organisms in question. A comparison of the viability of *Bact. coli* in a non-acid and in an acid-containing beverage is shown in table 3. It will be noted that the hydrogen-ion concentration of the latter is considerably greater than that of the former. The colon bacillus is killed much more speedily in the acid-containing beverage, the effect being especially marked at the higher temperature. Apparently the rapid de-

TABLE 2

*Viability of Bact. coli in carbonated tap water under pressure and with pressure released*

	TIME INTERVAL	PRESSURE 28 POUNDS PER SQUARE INCH (1.9 ATMOSPHERES) AT 24°C.		EXCESS CO <sub>2</sub> ALLOWED TO ESCAPE AT THE RESPECTIVE TEMPERATURES BEFORE INOCULATION	
Held at room temperature (22-24°C.)	At once	440,000	360,000	440,000	500,000
	24 hours	1,500	1,360	870	130
	3 days	13	18		36
	7 days	10 cc. + 5 cc. 0	10 cc. 0	10 cc. + 5 cc. 0	10 cc. + 5 cc. 0
	18 days	10 cc. 0	10 cc. 0	10 cc. 0	10 cc. + 5 cc. 0
Held at 1°C.	At once	500,000	390,000	300,000	540,000
	24 hours	34,000	14,700	8,200	10,300
	3 days	3,600	900	83	71
	7 days	100	88	1 cc. +	1 cc. +
	18 days	1 cc. +	1 cc. +	1 cc. +	1 cc. +

struction of *Bact. coli* in the acid-containing lemon soda is due mainly to the dissociation of the citric acid present, for in additional experiments in which this acid was omitted the death rate was found to be comparable to that of the non-acid vanilla soda. Also in this case the hydrogen-ion concentration had decreased from pH 3.0 to pH 4.0-4.4 by the omission of the citric acid.

Experiments with other acid-containing beverages have demonstrated that as the amount of acid is increased above that indicated in table 3, the more readily is the colon bacillus killed.



In one instance in which 0.156 per cent lactic acid (5 grains per 7-ounce bottle) was employed the numbers of *Bact. coli* dropped from several hundred thousand to several hundred per cc. in

TABLE 3

Showing the viability of *Bact. coli* in a non-acid and in an acid type of carbonated beverage

	TIME INTERVAL	VANILLA SODA (NON-ACID), PRESSURE 24 POUNDS (1.6 ATMOSPHERES) AT 21°C., pH 4.0-4.4		LEMON SODA (ACID TYPE), PRESSURE 19 POUNDS (1.3 ATMOSPHERES) AT 23.5°C., pH 3.0	
Held at room temperature (21-24°C.)	At once	132,000	136,000	215,000	160,000
	24 hrs.	20,900	32,700	900	200
	3 days			1 cc. negative	1 cc. negative
	4 days	140	20	10 cc. negative	10 cc. negative
	7 days	1 cc. positive	1 cc. positive		
	14 days	10 cc. negative	10 cc. negative		
Held at 1°C.	At once	140,000	127,000	225,000	215,000
	24 hrs.	68,000	46,000	115,000	144,000
	3 days			87,000	25,000
	4 days	17,200	13,500		
	7 days	11,100	11,200	6,800	4,900
	14 days	390	600	600	
	1 mo.	1 cc. positive	1 cc. positive	10 cc. negative	10 cc. negative
	2 mos.	10 cc. positive; 1 cc. positive	10 cc. positive; 1 cc. negative		

Composition of the above beverages:

*Vanilla soda*: 10 mgm. c.p. vanillin (0.0048 per cent) and 10 grams sucrose (4.8 per cent) per 7-ounce bottle (207 cc.).

*Lemon soda*: 0.5 cc. commercial lemon flavor, 3 grains citric acid (0.094 per cent), and 20 grams sucrose (9.6 per cent) per 7-ounce bottle.

Carbonated water added to make the finished beverage.

4 hours at 20°C. When plain non-carbonated tap water was substituted for the carbonated water in the acid beverages, the death-rate of *Bact. coli* remained practically the same. That is, in these instances the added acids are the chief causative agents

in the destruction of the colon bacillus, irrespective of the effect of carbon dioxide.<sup>1</sup>

Since it was found that *Bact. coli* is able to withstand carbonation for an appreciable period, the next step was to investigate the viability under similar conditions of several of the pathogenic members of the colon-typhoid group. In these experiments *Bact. paratyphosum* B and *Bact. typhosum* were used. It was at once apparent that both of these types are considerably less resistant to the destructive effect of CO<sub>2</sub> than is the colon bacillus. Table 4 presents results which are characteristic of a number of similar experiments. One point of particular interest is the persistence, at 1°C., of the last few surviving organisms. These were too few in number to be estimated by plating and their presence could be detected only by the cultivation of 10 cc. amounts in glucose broth. Additional experiments with acid-containing beverages have shown that in these the typhoid bacillus is killed almost instantly. Thus in a carbonated lemon soda containing 0.156 per cent lactic acid, *Bact. typhosum* decreased in numbers from an initial inoculum of 27,400 per cubic centimeter to 10 per cubic centimeter within one hour and after two hours its presence could not be detected.

It should be emphasized that throughout all of the foregoing experiments the water used for carbonation and for preparation of the various beverages was an ordinary city supply of low mineral content. Under certain conditions, as for example in carbonated water of high mineral content, it is possible that non-spore-forming organisms may remain alive for longer periods than those herein reported. This possible influence of certain inorganic salts upon the viability of microorganisms in a carbonated environment has not been studied in the present investigation.

<sup>1</sup> The usual methods of bacteriological analysis could not be applied when larger quantities of the highly acid beverages were to be examined. It was found that sufficient amounts of acid were carried over to the culture medium to cause a distinct increase in the H-ion concentration, sufficient, indeed, to effect a retardation or even complete inhibition of growth. By the use of larger quantities of broth in flasks, instead of the usual amounts ordinarily contained in test tubes, this difficulty was largely overcome.

TABLE 4  
Comparative viability of *Bact. typhosum* in carbonated and in plain (non-carbonated) tap water

TIME INTERVAL	CARBONATED TAP WATER, PRESSURE 25 POUNDS (1.7 ATMOSPHERES) AT 24°C.			CONTROL, PLAIN TAP WATER		
	Held at room temperature (22-24°C.)			Held at room temperature (22-24°C.)		
	Held at 1°C.			Held at 1°C.		
At once	45,000	36,500	44,100	27,000	42,000	63,000
24 hours	1 cc. plate negative	1 cc. plate negative	30	1 cc. plate negative	41,000	22,000
48 hours	10 cc. negative	10 cc. negative	10 cc. positive	10 cc. positive	27,000	12,000
4 days			10 cc. positive	10 cc. positive		
7 days			10 cc. negative	10 cc. negative		

Comparative viability of <i>Bact. typhosum</i> and <i>Bact. paratyphosum B.</i> in vanilla soda						
PRESSURE 23 POUNDS (1.56 ATMOSPHERES) AT 24°C.						
	B. typhosum			B. paratyphosum B		
	Held at room temperature (21-24°C.)			Held at room temperature (23-25°C.)		
	Held at 1°C.			Held at 1°C.		
At once	175,000	166,000	237,000	144,000	116,000	200,000
4 hours					800	
24 hours	40	270	10,300	21,600	3	7,300
48 hours	1 cc. plate negative	1 cc. plate negative	310	170	10 cc. pos.	860
3 days	10 cc. plate negative	10 cc. plate negative	10 cc. pos.	10 cc. pos.	10 cc. neg.	15
4 days	10 cc. negative	10 cc. negative	1 cc. neg.	1 cc. neg.	10 cc. neg.	
6 days	10 cc. negative	10 cc. negative	10 cc. positive	10 cc. positive	10 cc. neg.	10 cc. positive
7 days			10 cc. positive	10 cc. positive	active	active
10 days			10 cc. negative	10 cc. negative	10 cc. positive	10 cc. positive
14 days			active	active	10 cc. negative	10 cc. negative

The resistance of spores to the conditions described in this paper is of some interest when compared to that of *Bact. coli* and *Bact. typhosum*. The spores of both *B. mesentericus* and *Clostr. sporogenes* were found to be quite resistant, for after one month in carbonated water no reduction in numbers could be detected. In one experiment the spores of *B. mesentericus* survived in a citric acid beverage (pH 3.0) for one month with little, if any, diminution in numbers.

It must be stated emphatically that the results obtained in this investigation do not warrant the conclusion that water of a low sanitary quality can be used by the industry in the preparation of carbonated beverages, or that carbonation can be relied upon to destroy evidence of pollution. In many instances, particularly during the summer months, beverages are consumed within a few hours after their preparation and it is obvious that under these conditions pathogenic organisms, if originally present in the water, may survive carbonation and reach the consumer.

#### SUMMARY

Under the conditions of these experiments carbonation exerts a distinctly harmful effect upon the members of the colon-typhoid group and their period of viability in carbonated water is much shorter than that in plain tap water. The destructive effect of the CO<sub>2</sub> is especially marked at room temperature, 19° to 23°C., and less so at 1°C.

In a "non-acid" beverage, the organisms may persist for a slightly longer period than in carbonated water. In beverages containing 0.094 per cent or greater amounts of citric or lactic acids, the death-rate is very rapid and is apparently due to the effect of these acids, irrespective of the CO<sub>2</sub>.

*Bact. typhosum* and *Bact. paratyphosum* B are more readily destroyed by CO<sub>2</sub> than is *Bact. coli*.

The spore forms of a common aerobe, *B. mesentericus*, and of a common anaerobe, *Clostr. sporogenes*, were found to be quite resistant to carbonation, surviving one month at room temperature with no apparent diminution in numbers.

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# A BINOCULAR MICROSCOPE ARRANGED FOR THE STUDY OF COLONIES OF BACTERIA

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The binocular microscope arranged as described below has been used in this laboratory for several years with so much satisfaction and profit in the isolation of bacteria that a description of such a simple piece of apparatus may not be out of place.

Every one who has examined colonies of bacteria or other organisms on culture media by the use of direct illumination from below has experienced more or less difficulty. The medium is frequently too opaque to admit sufficient light, especially in the case of media containing blood; some very small colonies have the same refractive index, or so nearly the same refractive index, as their medium that observation may be very difficult even on transparent media. These difficulties are obviated and observation greatly facilitated by viewing the colonies in light reflected from the surface of the culture as is usually done in using a hand lens for this purpose. To make such observations with higher magnification and greater ease we arranged a binocular microscope with magnifications of ten and twenty diameters in the following manner. The tube and focusing apparatus was removed from the stage and base and attached to an independent support so that its optical axis was at an angle of  $45^\circ$  with the stage, though so arranged that the angle might be altered as desired (*A*, fig. 1). A small arc with a condensing lens was supported so as to project a beam of light at an angle of  $45^\circ$  with the stage and at right angles to the optical axis of the microscope. As this gave rather more light than was necessary a 75-watt "daylight" lamp was supported about

15 cm. above the stage and surrounded, except for a slit 2 cm. wide, with an opaque reflecting screen in such a position that a beam of light was projected to the stage at right angles to the optical axis of the microscope (*B*, fig. 1). A solid black stage provided support for Petri dish cultures or a grooved block placed on it provided for the support of tube cultures so as to bring the surface of the media parallel with the stage.

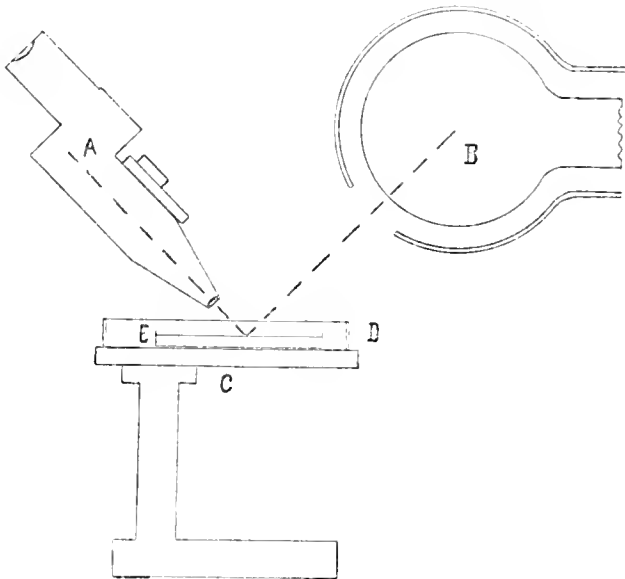


FIG. 1. A DIAGRAM OF A BINOCULAR MICROSCOPE ARRANGED FOR THE STUDY OF COLONIES ON THE SURFACE OF MEDIA

*A*, Binocular microscope tube and focusing apparatus supported on a base independent of the stage and at an angle of  $45^\circ$  with the stage; *B*, "daylight" 75 watt light surrounded by a screen except for a slit; *C*, solid black stage and base; *D*, glass screen to protect open plate cultures; *E*, Petri dish culture.

The focal distance of the binocular is sufficient to permit of the examination of tube cultures or Petri dish cultures with the cover left on. For the fishing of colonies from cultures which were to be further incubated a plain glass was supported 15 mm. above the stage by strips along three sides leaving the right



hand side open. An uncovered plate culture might then be slipped under this glass screen with very little danger of dust or breath contamination and at the same time permit the fishing of colonies, under microscopic observation, with a platinum wire. The most satisfactory platinum wires for this purpose were drawn out in the flame to a fine point which was then turned at a right angle about 1 mm. from the tip.

The chief value of the apparatus in this laboratory has been in the isolation of organisms producing very small colonies from material containing large numbers of other species, i.e., the isolation of *H. influenza* from sputums. It is a very easy matter to fish the smallest *H. influenza* or similar colonies of other species, or to remove half of such a colony and smear for microscopic observation and later to use the remaining half colony for the inoculation of subcultures.



AN INVESTIGATION OF AMERICAN STAINS  
REPORT OF COMMITTEE ON BACTERIOLOGICAL TECHNIC

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Early in 1920 the Committee on Bacteriological Technic was asked to look up the matter of biological stains at present available in America and to see what could be done toward standardizing them. At present it is difficult, if not impossible, to obtain the Grüber stains, and the American products are known to be variable. The impression has even been common that American dyes are generally unsatisfactory for staining.

Upon looking into the matter, the committee has found that many American stains are as good or even better than the old Grüber stains, except for certain special uses which their producers did not have in mind when preparing their products. Certain American producers of biological stains are trying very hard to put on the market an entirely satisfactory line of goods. The difficulty comes from the fact that the field is a small one, and so much competition has arisen that no one can make a satisfactory profit. The danger is that soon the American producers will all be driven out of the business and importation of stains will again be necessary. We do not desire to be dependent upon foreign production in this line, because of the great importance of stains in public health work and the possibility of another national emergency when importation will be impossible. To relieve the situation, therefore, some one manufacturer must be given enough support to make the business profitable for him. This means standardizing on one line of stains, either all produced by the same house, or if produced by different concerns, each particular stain coming from one manufacturer only.

<sup>1</sup> For the numerous collaborators in this work, see list at end of the report.

In order to survey the field it seemed necessary to test products from as many sources as possible. As the whole field is a large one, the attention of the committee at first was given to but three stains or groups of stains; fuchsin, methylen blue, and gentian violet. About thirty members of the Society,—to whom much credit is due,—volunteered to assist. Their names are given at the end of this paper; and without their hearty cooperation, the work would have been impossible. Our study is not complete yet, but a fairly satisfactory survey of the field has been made, so far as concerns fuchsin and methylen blue. The gentian violet situation is more complicated and further investigation is necessary.

Upon looking into the commercial situation, it proved necessary to obtain samples of the dyes from two different classes of dealers: the basic dye manufacturers and the so-called "manufacturers and standardizers" of biological stains. The former group are the manufacturers of general textile dyes, and embrace such concerns as the National Anilin Company, the duPont Company, the Calco Chemical Company, and Dicks, David and Company. The latter group generally buy their dyes from the former, make certain tests of them and put them on the market as biological stains, with or without modification; their chief function is standardization rather than manufacture. Representatives of the latter group are the Providence Chemical Laboratories, the H. S. Laboratories, the Coleman and Bell Company (formerly the National Stain and Reagent Company), and the Heyl Chemical Company. Samples from these various concerns were obtained through a jobber who had consented to cooperate in the work. This was done so that no concern would know the reason for which the samples were bought. To make unbiased opinions still more certain, the samples were distributed among the various investigators by number only, without reference to the names of the dealers.

Throughout this work, the committee has been in correspondence with all of the second group of dealers mentioned above, and with the Calco Chemical Company as well. There has seemed no purpose in getting in touch with the other basic

manufacturers, as the production of biological stains is a very small part of their business and the results of this work mean little to them. In general, hearty cooperation has been secured. All but two of the dealers have told us to feel perfectly free to publish the results. As these two are merely distributors of scientific supplies, and as it is difficult to tell the original source of the samples obtained from them or to learn whether their present supply is still the same, their names are suppressed here, using instead the designations A and B. For similar reasons the name of a third house of like character is suppressed, although in this case the committee was not asked to do so.

The list of dealers (other than these three) whose products have been examined is as follows. In this list the addresses are given for those firms who deal specially in biological stains.

Calco Chemical Company, 136 Liberty Street, New York City

Coleman and Bell Company, Norwood, Ohio

Dicks, David and Company

E. I. duPont de Nemours Company

Geigy Chemical Company

Goldin Biological Laboratories, Providence, R. I.

Harmer Laboratories Company, Lansdowne, Pa.

Heyl Laboratories, 437 Baretto Street, New York City

Holland Anilin Company

H. S. Laboratories, 6005 Girard Avenue, Philadelphia

H. Kohnstam and Company

Mallinckrodt Chemical Company

Merck and Company

National Anilin Company

Newport Chemical Works

Providence Chemical Laboratories, 51 Empire Street, Providence, R. I.

Williamsburg Chemical Company

Very few tests were made with the products of the Heyl Laboratories. This was merely because the Heyl products are sold under certain restrictions that make it difficult for jobbers to buy them. We could have obtained all the Heyl stains we

wanted direct from the company; but for the sake of fairness we did not want to buy this one brand of stains direct, while picking up the others on the open market. The jobber through whom we dealt had such delays in getting the Heyl samples that they were not ready at the time of our main tests. It might further be remarked that Mr. Heyl himself says that his crude dyes are generally obtained from abroad, as he does not believe the American products satisfactory.

One of the troublesome factors in the stain situation is due to confusion in nomenclature. It may happen that the same name is given to more than one product or that the same product is called by several names. Accordingly it seems worth while to discuss these three stains from the standpoint of their chemical composition and to list their synonyms. In the list of synonyms in each case, preferred names are given in bold-faced type.

#### FUCHSIN (BASIC)

##### *Synonyms*

**Rosanilin**  
Diamond fuchsin  
Magenta  
Rubin  
Anilin red

The formula of fuchsin, or rosanilin, is  $(\text{NH}_2 \cdot \text{C}_6\text{H}_4)_2 : \text{C} : \text{C}_6\text{H}_4 \cdot \text{NH}$ . Being a basic dye, this is ordinarily combined with an acid, and the dye with which we are familiar is the chloride  $(\text{NH}_2 \cdot \text{C}_6\text{H}_4)_2 : \text{C} : \text{C}_6\text{H}_4 : \text{NH} : \text{HCl}$ . Fuchsin proves the easiest to obtain in a satisfactory state of purity of any of the dyes we have so far investigated.

There are three chief uses of fuchsin in bacteriological work: general bacterial staining; staining for acid-fast qualities; and use in the endo medium for colon-typhoid differentiation. A satisfactory stain should give good results for all three purposes. In the present investigation, the acid-fast test has been most frequently used, but enough tests have been made for the other purposes to indicate the general utility of the different samples. Only two investigators have used the stains in the endo medium,

both of them using different technics; although one of these two (Castleman) made his tests on two different occasions, with a different technic each time.

Results were reported in two different ways. Some grouped the samples in three or four categories which it has proved possible to denote by four terms: excellent (E), good (G), fair (F), and unsatisfactory (U). Others listed the samples in the order of their excellence, in which case instead of placing them in these four categories, they have been numbered 1, 2, 3, etc., the low numbers indicating the best samples. These two sets of symbols are used in Table I, where the results of the different investigators with the different samples are listed.

It will be seen that there is some variation in the findings of the different investigators, although not as much as in the case of the other two dyes, reported below. When fuchsin was used as a stain it proved difficult to pick out any one sample or any two or three samples that were superior to the others. To make the comparison more definite, each of the four classes was given a numerical symbol, these numbers averaged, and the average converted back into the class symbol again, using the grades: E, E-, G+, G, G-, etc. By glancing at this average grade, it will be seen that the sample from the Providence Chemical Company ranks G+, none of the others ranking better than G. All but three of the others rank G.

The samples are rearranged in table 2 in the order of excellence, according to their average grade, this same table listing the number of times each sample was found excellent and the number of times found unsatisfactory. Only three samples were reported unsatisfactory by any one, namely those from dealers A, B, and C, each being so reported by two different investigators. These three were the only samples to have an average grade lower than G, and yet two of them were reported as *good* or *excellent* by some investigator. There seems no reason to feel that any of the samples, with the exception of these three, could not be successfully substituted for the Grüber product. The superiority of the Providence sample is so slight as to be of little significance.

TABLE 1  
Reports of the individual investigators on the samples of fuchsin

	AS STAIN INVESTIGATOR AND TEST USED										AVERAGE GRADE AS STAIN	AS INDICATOR ON INVESTIGATOR AND TEST USED				AVERAGE GRADE AS INDICATOR	
	Castleman, Ziehl stain	Castleman, Gram counter-stain	Boyd, Ziehl stain	Hill, Ziehl stain	Burnett, Ziehl stain	Meyers, Ziehl stain	Meyers, General stain	Craig, Ziehl stain	Roos, Ziehl stain	Wade, Ziehl stain		Pikens, General stain	Wade, Endo medium	Meyers, Endo medium	Castleman, first test, Endo medium		Castleman, second test, Endo medium
Calco.....	12	9	G	3	G	G		G		G		F	E	G	G	G-	
Dealer A.....	6	11	G	1	G	U		E		G		U	U	U	U	U	
Harmer.....	7	3	G	G	G	G		E		E		E	G	U	F	G-	
National Anilin.....	10	2	G	2	G	G		G		F		G	G	U	G	G-	
Newport.....	2	8	G	7	G	E		E		F		G	E	U	G	G-	
Providence.....	3	4	G	G	E	E		E		G		G	E	U	F	G-	
Coleman and Bell.....	5	1	G	5	G	G		G		F		E	G	U	G	G+	
Goldin.....	1	5	G	G	G	G		G		F		G	G	U	G	G-	
H. S.....	8	7	G	6	G	G		E		F		G	G	U	F	F	
Dealer B.....	11	10	G	4	G	E		U		U		F	F	U	G	G-	
Dealer C.....	9	12	G	9	U	U		U		F		U	U	U	U	U	
Dicks, David and Company.....	4	6	G	8	G	G		G		E		G	G	U	G	G	G-



When used in the endo medium, the samples were reported quite differently. The result plainly depends on the technic, some methods calling for too concentrated a solution of the dye to be decolorized by the amount of sulfite ordinarily used. The following formulae were used:

*Meyers:* 0.5 cc. of 10 per cent alcoholic fuchsin added to 10 cc. of 2.5 per cent sodium sulfite solution. This quantity added to 100 cc. of the agar. (Standard method of American Public Health Association.)

TABLE 2

*Summary of reports on fuchsin; samples arranged in order of excellence*

SAMPLE	AS STAIN				Conclusions	IN THE ENDO MEDIUM
	Number of tests	Number of times excellent	Number of times unsatisfactory	Average grade		
Providence.....	10	3	0	G+	Very good	Good
Calco.....	10	1	0	G	Good	Good
Coleman and Bell.	11	2	0	G	Good	Excellent
Dicks, David.....	9	0	0	G	Good	Good
Goldin.....	10	1	0	G	Good	Good
Harmer.....	8	2	0	G	Good	Good
H. S. Laboratories.	11	2	0	G	Good	Good
National Anilin...	9	2	0	G	Good	Good
Newport.....	9	2	0	G	Good	Good
Dealer B.....	11	2	2	G-	Fair to good	Good
Dealer A.....	9	1	3	F+	Fair to poor	Unsatisfactory
Dealer C.....	8	0	3	F-	Unsatisfactory	Unsatisfactory

*Castleman*, first test: 1 cc. of saturated alcoholic fuchsin added to enough 10 per cent sodium sulfite solution to decolorize. One cubic centimeter added to 100 cc. of agar.

*Castleman*, second test: 0.5 cc. of 1 per cent alcoholic fuchsin added to 1.5 cc. of 2.5 per cent sodium sulfite solution. One cubic centimeter added to 100 cc. of the agar.

The only sample giving universally satisfactory results in these tests is that of the Coleman and Bell Company. This finding has been corroborated by other results not included in the present series of tests. By using the proper technic, however, i. e., either the first or the third formula above, good results

were obtained with all the samples except those from dealers A and C. These two, it will be noticed, are the two poorest samples when used as stains. It has been found that even the Grübler product shows up no better in such tests, the Coleman and Bell sample being even more satisfactory than the German fuchsin

For these reasons our present recommendation in regard to fuchsin is in favor of the product of the Coleman and Bell Company. Although this does not show up quite as well in the staining tests as that of the Providence Chemical Company its rank is good, and its superiority as an indicator in the endo medium puts it almost in a class by itself. It is plain, however, that good results can be obtained with any of the samples except the three that stand lowest in table 2.

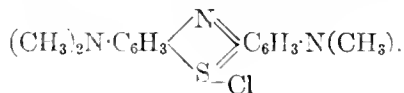
#### METHYLEN BLUE

##### *Synonyms*

Ethylene blue

Swiss blue

The chief confusion in regard to methylen blue lies in the fact that there are really two dyes on the market, one a pure chloride of methylen blue, the other a double salt, zinc and methylen blue chloride. The pure chloride has the formula:



The methylen blues which are not zinc-free are generally known as "methylen blue" or "methylen blue for bacilli." The free chloride, on the other hand, is specified in one of the following manners:

**Methylen blue, medicinal** (pure, zinc-free).

Methylen blue, U. S. P.

Methylen blue, BG.

The free chloride is soluble in both water and alcohol, the zinc salt is insoluble in alcohol.

Grübler's methylen blue for bacilli was apparently mostly zinc salt, this being the cheapest form of methylen blue available. It was not pure, however, and thanks, apparently, to the fact that small amounts of the zinc-free compound were present in it, it could be used in the Loeffler formula, which calls for 3 parts saturated alcoholic solution to 10 parts water. Unfortunately some of the preparations now on the market called "methylen blue for bacilli" are so nearly pure zinc salt as to be unsatisfactory for the Loeffler solution. On the other hand, some American stains labelled exactly the same are apparently nearly zinc-free, and are admirable for staining purposes, even in the Loeffler formula.

In the present series of tests, samples of both the zinc salt and the medicinal (zinc-free) dye were used. Fourteen investigators took part in the tests, and the samples were tested in several different ways. Four men, Healy, Hunter, Macy and Robertson, used the samples for staining dried milk smears according to the Breed method of examining milk for bacteria; Macy and Robertson used aqueous solutions of the stain samples, Healy and Hunter the Loeffler solution. Healy and Robertson also used their samples for staining ordinary bacterial cultures, and with the exception of Levine, all the other investigators used the stains for this purpose only, all employing the Loeffler solution, except Robertson who used a saturated aqueous solution. Levine tested the samples by using them in his cosine-methylen-blue medium for colon-aerogenes differentiation.

The test made by the writer was a special test made at the suggestion of Mr. Bell of the Coleman and Bell Company. Only those samples were selected for it which had already been found to give the best results. Three solutions of each were prepared: a 1 per cent aqueous, a 3:10 alcoholic aqueous (i.e., 3 parts saturated alcoholic solution to 10 parts distilled water), and a Loeffler solution (i.e., a 3:10 mixture using 0.0001 per cent NaOH instead of distilled water). Smears of a diphtheria culture were stained with each, allowing only about three seconds for staining and then washing under a tap. Mr. Bell suggested this as the most severe test he knew, and one which his methylen blue for bacilli was required to meet.





TABLE 4

Summary of reports on methylen blue; samples arranged in order of excellence

SAMPLE	AS STAIN				Conclusions	IN THE LEVINE MEDIUM
	Number of tests	Number of times excellent	Number of times unsatisfactory	Average grade		
<i>For bacilli:</i>						
Coleman and Bell	9	5	1	E-	Very good	Excellent
Heyl	2	2	0		Very good	Not tested
National Anilin	7	1	1	G-	Fair to good	Fair
Calco	9	1	2	F+	Fair	Good
Harmer	7	1	2	F+	Fair	Unsatisfactory
H. S. Laboratories	6	0	2	F+	Fair	Unsatisfactory
Geigy	7	0	3	F	Fair to poor	Unsatisfactory
Holland Anilin	6	0	2	F	Fair to poor	Good
Kohnstam	8	1	4	F	Fair to poor	Excellent
Providence	6	0	2	F	Fair to poor	Good
Dealer A	7	0	3	F-	Unsatisfactory	Excellent
* Dieks, David	8	0	6	U	Unsatisfactory	Unsatisfactory
<i>Medicinal:</i>						
Calco	7	5	0	E-	Very good	Good
Dealer C	7	5	0	E-	Very good	Good
Williamsburg	6	3	0	E-	Very good	Excellent
Coleman and Bell	7	4	0	G+	Very good	Good
Heyl	2	0	0		Very good	Not tested
Providence	9	2	0	G+	Very good	Unsatisfactory
Goldin	10	2	0	G	Good	Fair
H. S. Laboratories	9	4	0	G	Good	Fair
Merek	8	0	0	G-	Fair to good	Unsatisfactory
* Dealer A	7	0	2	F	Fair to poor	Excellent
* Harmer	8	1	5	F-	Unsatisfactory	Fair
* Mallinkrodt	9	0	5	F-	Unsatisfactory	Unsatisfactory
* Dealer B	5	0	3	F-	Unsatisfactory	Good

\* These samples dissolve milk smears when used by the Breed method.

The results are listed in table 3, in which the same set of symbols is used as in table 1. A summary of the results is given in table 4. The most apparent fact is that the best results in staining have been obtained with the zinc-free dye. This is presumably because of the insolubility of the zinc salt in alcohol,

as Macy and Robertson, who used aqueous solutions, obtained essentially as good results with the zinc salt. The striking exceptions to the inferiority of "methylene blue for bacilli" are the samples from Coleman and Bell, and from the Heyl Chemical Company. After completing the test, it was learned that these two samples, although not labelled medicinal, and not claiming to meet the U. S. P. requirements, are fairly free from zinc, having been especially purified and corrected for bacterial staining.

Of the samples of medicinal methylene blue, six stand at the top, as a stain, with the honors quite evenly divided between them: namely, Caleo, dealer C, Williamsburg, Coleman and Bell, Heyl, and Providence. Almost as good are the Goldin and H. S. samples. Of these eight companies, we find that the Caleo Chemical Company, the Coleman and Bell Company, the Heyl Laboratories, the Providence Chemical Laboratories, and the H. S. Laboratories are making special efforts to put out a pure product well adapted to both staining and therapeutic uses. It is plain that they are succeeding in this, so far as staining properties are concerned, and provided the quality of their products is maintained any of them may be used as a stain without question.

One fault found with certain samples of methylene blue is that they have a tendency to dissolve milk smears off the slide when used for the Breed method. The chemical explanation of this phenomenon has not been obtained. The samples giving this trouble were the zinc salt sample from Dicks, David and Company, and the medicinal samples from Harmer Laboratories, Mallinkrodt Chemical Company, and from dealers A and B. It is interesting to notice that these same samples have been graded lowest according to their staining properties. They are plainly not to be recommended.

There is little correlation between the results of the staining tests and those obtained by Levine with the eosine-methylene-blue medium. The samples from dealer A, for instance, which stood low as regards staining properties were among the four

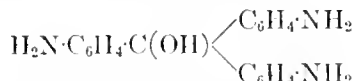
best samples in this medium. The medicinal sample from the Providence Laboratories, on the other hand, proved good for staining but unsatisfactory in this medium. The five samples grading good or very good as stains and also proving good or very good as an indicator were the two Coleman and Bell samples and the medicinal samples from the Calco Chemical Company, Williamsburg Chemical Company, and from dealer C. Those samples proving unsatisfactory were condemned because of toxicity. Strange to say, as many medicinal samples as zinc salt samples proved toxic, although for medicinal purposes the zinc salt alone is condemned on account of toxicity. In fact, for this medium, the zinc salt seems to prove almost if not quite as good as the free chloride.

There can be no question that, so far as our data go, one of the five samples just mentioned as satisfactory for both purposes should be recommended. The Heyl samples seem to rank with these as to staining properties, but for the reasons mentioned in the introduction, could not be included in the early tests, so we do not know how satisfactory they would prove in the Levine medium. We have not learned the original source of the sample from dealer C. The Williamsburg sample was labelled medicinal, but proves not to be entirely zinc-free. As a result, the composition of this product may well have been changed by the present time to meet U. S. P. requirements, and one could not count on getting another sample like the one tested. The two Coleman and Bell samples and the Calco medicinal sample all give very good results, and are produced by companies we believe to be reliable and to supply nothing but distinctly American products. Both companies we understand to manufacture their own methylen blue from American-made crudes or intermediates. Any of these three samples can be used with entire confidence of obtaining good results. Of these three, the Coleman and Bell "methylen blue for bacilli" has two points especially in its favor: it is not quite so expensive as the U. S. P. preparations, and it is the best of these three samples in the Levine medium.



## GENTIAN VIOLET

The term gentian violet is at present very indefinitely used. Grüber apparently originated it, applying it to a certain mixture of dyes all closely related chemically and having similar staining properties. These dyes belong to the pararosanilin series. The pararosanilin base is considered to have the formula:



In this formula it will be noticed that there are six hydrogen atoms attached to the three amino-nitrogen atoms. These six hydrogen atoms may each be replaced by a methyl group ( $\text{CH}_3$ ), and ethyl group ( $\text{C}_2\text{H}_5$ ) or even by a benzyl group ( $\text{C}_6\text{H}_5$ ). The compounds of most importance in gentian violet are those with four, five and six methyl groups respectively, known as:

tetramethyl-pararosanilin  
 pentamethyl-pararosanilin  
 hexamethyl-pararosanilin

In these three compounds, the greater number of methyl groups present the deeper is the shade of violet. Thus the tetramethyl compound is a reddish violet while the hexamethyl compound is a bluish violet. The introduction of a benzyl group tends to darken the shade still further. The tetra- and pentamethyl compounds are seldom prepared in a pure state, but the hexamethyl compound, under the name of crystal violet, is readily obtainable in fairly pure form. Grüber's gentian violet, besides these compounds, is supposed to have contained other rosanilins, but these three methylated pararosanilins were the most important constituents. American gentian violets are sometimes pure crystal violets, sometimes some other dye of this series, and sometimes various mixtures of the above mentioned dyes. The term gentian violet is not used by the dealers in textile dyes nor by their dye chemists. This stain is ordered only by the biologist, and hence it is furnished only by biological supply houses and some dye manufacturers that specially cater to the biologist.

These stains may be grouped in the following three categories:

## I

**Methyl violet**  
Methyl violet B  
Methyl violet 2B  
Methyl violet BS  
Methyl violet BN  
Methyl violet BO  
MethylvioletBBN  
Dahlia B

Under these designations are sold certain mixtures of the tetramethyl and pentamethyl compounds, in varying proportions, sometimes also containing the hexamethyl compound as well. The letters following the name are used in the trade to indicate the shade, methyl violet B and methyl violet 2B, for example, each being progressively deeper in shade than plain methyl violet. The trade designations do not refer to chemical composition except in so far as the deeper shade indicates a more highly methylated compound.

## II

**Benzyl violet**  
Methyl violet 5B  
Methyl violet 6B  
Methyl violet 7B

These dyes are benzylated compounds, generally mixed with some of the purely methyl compounds above mentioned. The number of B's in the trade designation indicates the depth of violet produced in dyeing.

## III

**Crystal violet**

This dye is the pure hexamethyl compound. It is the most readily obtained in pure state of the whole group, and there is less confusion in regard to its composition than in regard to any of the others.

For the present series of tests, samples of the following were collected: Methyl violet, methyl violet B, methyl violet 2B, methyl violet BS, methyl violet 6B, crystal violet, and gentian violet. There were over thirty samples in the entire lot, too many, it was felt, to send to any one investigator for the first test. Accordingly a more or less indiscriminate selection was made, so that each investigator had about twelve samples to test, generally one or two of each type of dye. They were asked to use the samples for the Gram stain, each man to employ his own technic, but to give the exact procedure used. As it turned out, scarcely any two men used the same technic, so the results varied greatly. The plan is to run another test with a standard technic. At present so few men have reported on any one sample of dye and such various techniques were used that the results are not regarded as having more than a general significance. It would not be fair to judge the individual samples on the basis of this preliminary work; so in this report the names of the manufacturers and distributors are not given. Each sample is denoted by number alone, each number indicating a certain business house. Results are given in table 5.

It was not expected when the work was started that the compounds of lower methylation would prove satisfactory for the Gram stain. It was somewhat of a surprise, therefore, to find that out of 84 tests of individual samples of methyl violet, methyl violet B and methyl violet 2B, there were 28 reports of good results, and one report of excellent results. Nevertheless, out of these same 84 tests, there were 36 reports of unsatisfactory findings, such a high percentage in comparison with the results obtained with the higher methylated compounds that no further work is to be done with these dyes.

The dyes called anilin violet, methyl violet BS, methyl violet 6B, and crystal violet, give in general as good results as "gentian violet" and sometimes better. There is some variation, however, in the products of different manufacturers, but on the basis of these few tests, none can be condemned. According to present indications, crystal violet can be substituted very successfully for gentian violet in the Gram stain. Some manufacturers at





present make this substitution for us, sending crystal violet when gentian violet is ordered.

The chief cause of variation in results seems to be due to the differences in technic. This shows plainly the need of standardizing the Gram stain, a task proposed to this committee some time ago. It is hoped as the result of the present work to accomplish this. Apparently the Stirling technic does not always work with these American products, as they are generally more concentrated than Grüber's gentian violet. Grüber's stain is well known to have contained a large amount of dextrin. The investigators who have used one of the methods calling for a carbolic solution seem to have obtained the best results; although one man who has compared a carbolic solution with the anilin-sulphate solution of Atkins, reports even better results with the latter.

In conclusion it can be said that several of the American gentian and crystal violets are as good, and some of them better than the Grüber product, when the Gram stain is used as a criterion. It is still too soon to endorse the product of any one manufacturer or distributor, but good results can apparently be counted on with any of the following:

Methyl violet 6B .....	Coleman and Bell, Heyl, or H. S.
Crystal violet.....	Coleman and Bell, Harmer, or Providence
Gentian violet.....	Coleman and Bell

It is suggested that anyone wishing to order before definite recommendations are made by the committee specify one of these seven products.

#### GENERAL RECOMMENDATIONS

There is at present so much confusion in this field that the biologist generally has little idea what actual dye he is buying under any given name. One practice that has contributed to this is that distributors buy the stains in bulk and then repack in small containers bearing their own label without mention of the name of the manufacturer or standardizer. Sometimes the distributor renames the stain on his own responsibility. There

is no objection to the distributor's name occurring on the label, provided the stain is designated by the original name, followed by the name of the manufacturer or standardizer from whom it was obtained. Our chief recommendation at present is therefore that everyone buying stains from dealers in general laboratory supplies insist that the source of the stain as well as the distributor's name be printed on the container. It is our hope that the distributors may coöperate with us in doing this.

When it comes to selecting between the different brands of stains on the market, it must be recognized that the number of dyes so far tested are so few that no general recommendations can be made as yet to apply to all bacteriological dyes. The present work, however, indicates that the products of Coleman and Bell, the Calco Chemical Company, the Providence Chemical Laboratories, and the H. S. Laboratories, all rank well. The managers of the companies are willing to coöperate, and they are all putting on the market stains that there is every reason to believe are of strictly American make. In particular, we would call attention to the extremely good showing of the Coleman and Bell (formerly National Stain and Reagent Company) products. This company, moreover, has been good enough to furnish the committee with fairly definite specifications for those dyes so far tested, an important point because one of the chief matters to be kept in view is the permanency of the supply of stains. There is every reason to expect that the Coleman and Bell Company, given adequate support by the users of stains and government protection against foreign products, will be able to continue in the business; but if it should not, much of the information obtained by this company would be at the disposal of our committee to use in duplicating their products. On account of their willingness to coöperate and their eagerness to be of service to bacteriologists, all of the Coleman and Bell products—even those not yet tested—deserve a thorough trial.

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# JOURNAL OF BACTERIOLOGY

## Contents

### NOVEMBER, 1921—VOL. VI—No. 6

- GEORGE E. HOLM AND JAMES M. SHERMAN. Salt Effects in Bacterial Growth. I. Preliminary Paper.
- HILDA HEMPL HELLER. Suggestions Concerning a Rational Basis for the Classification of the Anaerobic Bacteria. Studies in Pathogenic Anaerobes. IV. I. Preliminary Paper.
- J. HOWARD BROWN. Hydrogen Ions, Titration and the Buffer Index of Bacteriological Media.
- J. E. RUSH AND G. A. PALMER. On Decreasing the Exposure Necessary for the Gelatin Determination.
- HAROLD MACY. Chart of the Families and Genera of the Bacteria.

### SEPTEMBER, 1921—VOL. VI—No. 5

- G. P. PLAISANCE AND B. W. HAMMER. The Mannitol-Producing Organisms in Silage.
- HILDA HEMPL HELLER. Principles Concerning the Isolation of Anaerobes. Studies in Pathogenic Anaerobes. II.
- JOHN F. NORTON AND MARY V. SAWYER. Indol Production by Bacteria.
- AUGUSTO BONAZZI. On Nitrification. IV. The Carbon and Nitrogen Relations of the Nitrite Ferment.
- TH. THJOTTA AND ODD FALSEN SUNDT. Toxins of Bact. Dysenterias, Group III.

### JULY, 1921—VOL. VI—No. 4

- LAURA FLORENCE. Spiral Bodies in Bacterial Cultures.
- JAMES M. SHERMAN. The Cause of Eyes and Characteristic Flavor in Emmental or Swiss Cheese.
- G. J. HUCKER. A New Modification and Application of the Gram Stain.
- LOUIS J. GILLESPIE. Color Standards for the Colorimetric Measurement of H-Ion Concentration.
- HARRIET LESLIE WILCOX. The Effect of Pepton upon the Production of Tetanus Toxin.
- K. G. DERNEY AND J. BLANC. On the Growth and the Proteolytic Enzymes of Certain Anaerobes.

### MAY, 1921—VOL. VI—No. 3

- S. ORLA-JENSEN. The Main Lines of the Natural Bacterial System.
- KAN-ICHIRO MORISHIMA. Variations in Typhoid Bacilli.
- FREDERICK A. WOLF AND I. V. SHUNK. Solid Culture Media with a Wide Range of Hydrogen or Hydroxyl Ion Concentration.
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EDITOR-IN-CHIEF  
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DEVOTED TO THE ADVANCEMENT AND DISSEMINATION OF KNOWLEDGE IN REGARD TO THE BACTERIA AND OTHER MICRO-ORGANISMS

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## CONTENTS

F. C. HARRISON. Our Society.....	119
VICTOR BURKE. Notes on the Gram Stain with Description of a New Method.....	159
BARNETT COHEN. Disinfection Studies. The Effects of Temperature and Hydrogen Ion Concentration upon the Viability of <i>Bact. coli</i> and <i>Bact. typhosum</i> in Water. IS3	
SELMAN A. WAKSMAN. Microorganisms Concerned in the Oxidation of Sulfur in the Soil. I. Introductory.....	231
SELMAN A. WAKSMAN AND J. S. JOFFE. Microorganisms Concerned in the Oxidation of Sulfur in the Soil. II. <i>Thiobacillus Thiooxidans</i> , a New Sulfur-oxidizing Organism Isolated from the Soil.....	239
E. B. FRED AND W. H. PETERSON. The Production of Pink Sauerkraut by Yeasts.....	257
COSTANTINO GORINI. Studies on the Biology of Lactic Acid Bacteria: a Summary of Personal Investigations.....	271
L. D. BUSHNELL. A Method for the Cultivation of Anaerobes.....	277
L. D. BUSHNELL. Influence of Vacuum upon Growth of Some Aerobic Spore-Bearing Bacteria.....	283
H. R. BAKER. Substitution of Brom-Thymol-Blue for Litmus in Routine Laboratory Work.....	301
W. A. WALL AND A. H. ROBERTSON. The Use of Domestic Methylene Blue in Staining Milk by the Breed Method.....	307

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## OUR SOCIETY<sup>1</sup>

F. C. HARRISON

*Principal, Macdonald College, P. Q., Canada*

### INTRODUCTION

The constitution of our Society states that the object of the Society shall be the promotion of the science of bacteriology, the bringing together of American bacteriologists, the demonstration and discussion of bacteriological methods, and the consideration of subjects of common interest: Thus, in accord with other scientific societies, its ultimate object is to make life a better thing than it is, to help in the service of man, and to attempt in some small measure to attain what Aristotle in Book III of *The Republic* has expressed that:

Our youth will dwell in a land of health, amid fair sights and sounds and receive the good in everything; and beauty, the effluence of fair works, shall flow into the eye and ear like a health giving breeze from a pure region and insensibly draw the soul from earliest years into likeness and sympathy with the beauty of reason.

To accomplish these benefits, our work lies ready to our hands, but our strength may be the greater and our faith the firmer if we spare a moment from present toils to look back upon the achievements of the past, to gather strength and encouragement before confronting the future.

The Society was founded in 1900, under the presidency of the late Prof. W. T. Sedgwick, a great teacher, an inspiring investigator and a kindly spirit that radiated good will and courteous consideration to others. In the two decades just past the Society has increased in stature and in wisdom and in favor with fellow workers.

<sup>1</sup> Address of the President at the Twenty-third Annual Meeting of the Society of American Bacteriologists.

The membership has grown from fifty in 1900 to over one thousand in 1921. The Society has tasted the joy that springs from labor, and perhaps its greatest achievement has been the establishment of the JOURNAL and ABSTRACTS which are to our workers ports and happy havens indeed. Further achievements of happy memory and daily use to the teacher and investigator are the Society's card, the standard methods of many routine proceedings; some order out of the chaos of bacteriological nomenclature; and the commencement of work on standardizing materials and methods.

The recital of the past is an augury for the future. Can we, at this time, suggest a policy for our Society? Are we able to plan a coöperation of efforts which will interest all members, for each to give something of himself for the good of the Society, and for the benefit of human life and effort?

"The keen spirit seizes the prompt occasion—makes the thought start into instant action, and at once plans and performs, resolves and executes."

May I, therefore, take this opportunity of placing before you a few thoughts regarding the future.

#### I. STEADY CAMPAIGN FOR MEMBERSHIP, AND THE ESTABLISHMENT WHERE POSSIBLE OF LOCAL BRANCHES

Thanks to the activity of Dr. Ayers and his committee, there has been this year a large increase in membership. But we must have more, if the plans outlined for a larger journal are to be carried out; a large membership is fundamental and when obtained many other things will follow in consequence.

We have started a number of local branches. No scheme offers better prospects of success for keeping the interest in the Society between annual meetings. Every endeavour should be made to increase these local organizations for, besides interest, they afford opportunity for social meetings, arouse a feeling of professional solidarity and permit of more frequent contributions to our science. Further, by enlarging the field from which members are drawn, the danger of narrow specialization is avoided, and members of the local branches will be given a broader outlook.



## II. IMPROVEMENT OF THE JOURNAL AND ABSTRACTS

When so much has been done in establishing these two important journals and bringing them to their present state of excellence, any drastic criticism would be a task of supererogation. I mention this matter, however, because our Secretary, in his circular letter dated October 25, 1921, stated that the Editor must have more space in order that papers offered for publication may appear more promptly. In order to make the *JOURNAL OF BACTERIOLOGY* a monthly periodical, there must be an increase of membership to 1500, or else other means must be adopted, and several alternate suggestions are set forth.

All of us would like to see the size of the *JOURNAL* increased, and regular publication guaranteed; these benefits can best be obtained only by an increase of membership. If our publications maintain a high standard of excellence, their worth will ensure more subscriptions from foreign countries.

Each member should be personally interested in the success of these journals, and should see that they attain a wider field of usefulness. Judicious and frequent suggestions to libraries that do not take them, bringing them to the notice of medical men and others interested in cognate subjects would help to advertise and undoubtedly secure subscribers.

## III. PROVISION FOR CRITIQUES AND RÉSUMÉS

*ABSTRACTS* at present is filled with references and short résumés of work done by bacteriologists all over the world.

I suggest that it would be of considerable interest to arrange for critiques or comprehensive résumés on many subjects, to be written by students of particular groups. For example: a comprehensive summary of the literature on the decomposition of cellulose; botulism; the carrier of infection, etc. Such reviews would be of great value to students, save much time in hunting up references, and bring the subject up to date. If written in a judicial spirit, the writer would be able to size up the situation, and give an appreciation of the subject as a whole.

If possible, such contributors should be paid.

## IV. CARD INDEX COMPILED FROM ABSTRACTS

The Library of Congress prepares and issues a card index of books and periodicals. The United States Office of Experiment Stations issues a card index of all its publications and that of the experiment stations. Would it be possible, and would it fill a need, if the editors of ABSTRACTS prepared a card index of the papers abstracted? The basis of subscription would have to be the individual card. Laboratories might arrange to subscribe according to the titles and sub-titles of the table of contents of ABSTRACTS.

General bacteriology should interest all. Agricultural colleges would naturally desire the card index for dairy, soil, and plant bacteriology. Public health laboratories should be interested in water, sewage and food bacteriology and health board laboratory methods and so on.

Prompt service as regular as the issue of ABSTRACTS would be possible.

How many would be willing to subscribe for such a service?

## V. COÖPERATION BETWEEN OUR SOCIETY AND THE AMERICAN PUBLIC HEALTH ASSOCIATION

In some regards our Society and the laboratory section of the American Public Health Association cover similar subjects. Both organizations have attempted to standardise technique and methods, both have published so-called standard methods. Members of our Society have been prominent in the American Public Health Association and vice versa. I suggest that some form of coöperation be instituted which would prevent any useless duplication of work. The two societies should work together, for in unity there is strength, and projects to be initiated by each might well be considered jointly, not necessarily by the whole society in convention, but at any rate by responsible committees of each.

## VI. A COMPREHENSIVE STUDY OF METHODS AND MATERIALS

Valuable work has been done by committees of the Society who have given their labor and time ungrudgingly in order to work out methods or examine materials. I mention this here because I believe that a more comprehensive survey of methods and material would be of great value. In biological problems we cannot hope for the minute exactness of the chemist, but the chemist has his standard and authorised methods, which he dare not depart from. When a method is being improved or a new one instituted a committee and official referee is appointed, and a thorough test, often extending over years, is given. It seems that such methods would be helpful in bacteriological technique and help to ward aside many criticisms that are at present levelled at us. The Society might add to the committees already at work, and assign more problems to be worked out for the common good.

## VII. BUREAU OF EMPLOYMENT

The Chemists Club of New York city has managed an employment agency since 1913 with marked success. I have frequently availed myself of its services with satisfaction to all concerned, and I suggest that our society might organize and run a similar bureau. If this employment bureau were incorporated as a "membership corporation" no person could benefit by the profits but any such accruing would go to the Society. Such an organization, if carefully organized and conservatively run, should be of great service to those in administrative positions, or heads of departments desiring to obtain assistance, not to mention those aspirants to bacteriological fame who desire to place a foot on the first rung of the ladder of employment opportunity. Certain large employers of trained bacteriologists, such as the health departments of large cities, could file their specific needs with the bureau, and these organizations could be kept informed of any men coming on the list who were specially fitted for their particular line of work.

## VIII. A BACTERIOLOGICAL MUSEUM

The American Museum of Natural History in New York has maintained a bacteriological museum under the skilled supervision of Prof. C.-E. A. Winslow. This has been a valuable asset to members of the Society, and should have support. Some of the older members may remember the Museum which Kral maintained in Prag, and the fine series of museum specimens and photographs he used to prepare. Kral's collection was very helpful to teachers and investigators, and has now been moved to Vienna.

The Society should have a museum, which should serve as a repository for all type species of organism described in our literature; further, I suggest that part of the work of such a museum would be to keep up the pathogenicity of organisms of economic importance, or those used for teaching. For several years I have sought plant pathogens of known virulence, for class work, but have received organisms devoid of pathogenicity, to the disappointment of the classes, and the possible loss of faith in one's veracity.

If the American Natural History Museum will undertake this work, nothing more need be done by the Society, save to let all members know what services the museum can render. If, however, some measure of support is necessary, I trust that the Society will investigate in what way the interests of its members can best be served, and make proper business arrangements with the Museum.

I suggest also another function for a museum, that of a

## IX. BACTERIOLOGICAL CLEARING HOUSE

You are all aware of the functions of a clearing house in a large city, an organization the members of which meet daily and pass through the various cheques and transactions of many banks.

Something similar would be valuable to the bacteriologist. I presume that many of you have had similar experiences to my own. Working on a particular problem, or doing some routine work, such as water or milk analysis, you find occasionally an

organism that attracts your interest by some peculiarity or abnormality. You isolate it, and put it by, with the intention that at some convenient season you will investigate it further, but alas, procrastination is the thief of time; the convenient season does not come, and the organism probably dies of exhaustion, starved by inattention to its material needs.

Now, if we had a clearing house the course would be different. You would say, candidly, I have not the time to work out this organism; it is interesting on account of its morphology or some peculiarity of its culture; I shall send it to the clearing house with a note as to where it was found, and its peculiarities. It is, therefore, duly dispatched, and on arrival at the clearing house, those in charge will read the letter and note the peculiarities, and will say, send this to John Doe, he is interested in this line and is working on this particular group, or investigating this irregularity, etc., or failing a student of this group, the organism might be investigated by those in charge of the clearing house.

This is a function that a well equipped and adequately staffed museum might undertake—possibly the American Museum of Natural History might institute a department charged with such work. If this were possible it would be a great asset to our Society. Failing such an organization, it might be possible to arrange for some distribution through the Journal.

#### X. A NEW DETERMINATIVE BACTERIOLOGY

Those of us who teach and those who are engaged in general or systematic studies, know the difficulties experienced in determining species.

Frantic search of Chester, Migula, Matzschita, of monographs here, of periodicals there, often fail to give any information or assistance on the points we desire, and then we realise the inadequate nature of our descriptions and our classifications, for they are numerous.

The Society, through the interest of some of its members, has taken cognizance of the chaos in classification and has adopted certain revisions which are helpful.

The Society also has a chart that has undergone a number of revisions, and which in its present form represents what is considered necessary for a proper description of an organism. These two contributions are excellent, but not enough. We want full descriptions as per Society chart of all known organisms, and we want them properly named and classified according to our latest classification. Here then is a splendid task for the Society, a task seemingly of great magnitude, but with proper organization and coöperation we should be able to surmount all obstacles, and produce a new determinative bacteriology approved by the Society, and all interested in bacteriology. Such a publication from its intrinsic value would find a place in every laboratory and succeeding generations of students and workers would rise up and call us blessed.

We have a membership of a thousand. If each one would pledge himself to give a full description of an organism assigned to him, what a magnificent start it would be. I trust the Society will accept this suggestion and formulate the necessary committees.

#### XI. TEACHING PROPAGANDA FOR BACTERIOLOGY

A subject, the study of which may not only serve on account of its educational value by enlarging our knowledge of nature and training the powers of observation and judgment, but also because of its sheer practical utility as the servant of medicine, pathology, sanitation, industry, agriculture, and household life; should have a well defined place in all our colleges and universities, and possibly in secondary schools. Yet we find that it is not mentioned in the curriculum of eight of our agricultural colleges. In many medical colleges it takes a subordinate place, and in many institutions having comprehensive courses in botany and zoölogy no course is given in bacteriology. Surely this is not right. Several past presidents of our Society have spoken about the value of the subject from the educational and practical aspects, notably the late Prof. W. T. Sedgwick and Professors Bergey, Marshall and Jordan, and there are excellent references in our literature as to the place the subject ought to

hold in our halls of research and learning. Therefore, all members of this Society should carry on incessantly and enthusiastically an active propaganda for bacteriology, for more bacteriology, and for still more bacteriology until the subject becomes more widespread in its benediction and embraces all classes and all institutions of secondary and higher learning.

A subject, which has achieved so much for the relief of suffering and the prevention of disease and whose effective progress and definite mission can be measured almost daily merits the early attention of the pupil and student.

Let us remember the words of the Greek philosopher, Prodicus, "That which benefits human life is God."

#### CONCLUSION

In conclusion, let me state that I have made these suggestions in the hope that they will be freely discussed by our members. We have a live Society, we are all interested in a subject that has advanced in the last forty years by leaps and bounds, there is much to investigate and nothing can provide an investigator with quite so pleasant a decoy as the persuasion that his chosen theme is in the nature of virgin soil. Here is a true adventure of the spirit, for he is adding a few sovereign grains of gold to the riches of his science for the enrichment of present life.

If little labour little are our gaines;  
Man's fortunes are according to his paines.

—*Herrick.*





# NOTES ON THE GRAM STAIN WITH DESCRIPTION OF A NEW METHOD<sup>1</sup>

VICTOR BURKE

*From the Department of Pathology, Stanford University, San Francisco*

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An examination of the literature concerning the Gram method of staining bacteria and of the methods of making the stain in various laboratories reveals the fact that a successful stain, i.e., one giving sharp differentiation between Gram positive and Gram negative organisms, can be obtained by several methods. A comparison of these procedures indicates that in a successful method each step is properly coordinated with and influenced by the other steps in the process. One step can be modified if other steps are also properly modified. This fact has been recognized and made use of in the various processes employed to improve or shorten the process of making the Gram stain.

Of all the various methods advanced none has successfully eliminated the personal factor. A satisfactory stain, especially of pus smears, depends to some extent upon the skill of the operator. Any one procedure has to be more or less modified depending upon the type of smear to be stained. For this reason the untrained assistant can not always be depended upon to produce satisfactory results by following any method so far advanced.

The skill of the operator, aside from his knowledge of the organisms to be examined, depends, upon the proper control of decolorization and the ability to detect any defect in the primary stain used.

<sup>1</sup> Aided by funds from the United States Interdepartmental Social Hygiene Board for Research in the Prevention and Cure of Venereal Diseases.

The control of the decolorizer requires experience. Some of the methods used advocate exposure of the smear to the decolorizer for a definite period of time, other methods recommend exposure to the decolorizer as long as any of the stain comes out. The skilled technician modifies both of these methods as the occasion arises. The time of exposure to the decolorizer must vary with the type of smears to be examined and depends upon the excellence of the violet stain and decolorizer used and upon the nature of the treatment accorded the smear between the removal of the iodine solution and the application of the decolorizer. It has not heretofore been recognized that the extent of the blotting and accompanying drying of the smear after the iodine solution greatly influences the decolorization of the Gram negative organisms. The process of blotting after the iodine constitutes one of the two critical steps in the Gram stain and will be discussed in detail later. Aside from the blotting after the iodine and the control of the decolorizer, the balance of the process can be carried on by an untrained assistant.

No one method so far advanced has been shown to be distinctly superior to all the other methods. The Committee on the Descriptive Chart of the Society of Bacteriologists has not yet selected any one method as the standard method to be used in the study and description of pure cultures. This committee has presented three methods for the consideration and criticism of the members of the Society. (Conn, H. J., 1919; Atkins, K. N., 1920.) While not recommending any one complete method it does recommend that 100 per cent alcohol be used as the decolorizer, that the films be made with distilled water and that the gentian violet, iodine solution and alcohol be drained or blotted off but not washed off of the film.

The different methods so far advanced vary in the making of the solutions, the strength of the solutions and the time periods of exposure. The most important modifications of the method as presented by Gram have resulted from efforts to improve the primary stain by increasing the penetration and intensity of the stain and the permanency of the solution. In most cases increasing the intensity of the stain by the use of mordants such

as anilin oil and phenol results in a loss in the stability of the solution. The stability of the solution has been found to vary with the method of mixing the ingredients together. The original anilin gentian violet as described by Gram may deteriorate in a few days. Sterling's modified solution lasts several months. Other modifications are said to keep indefinitely. Perhaps the most promising of these is the one recently described by Atkins (1920) in which anilin sulphate replaces the anilin water in the primary stain and NaOH is added to the iodine solution.

One of the objections to the use of the Gram stain has been the unsatisfactory or unstable nature of this primary solution. Until the discovery of Jensen (1912) that an aqueous solution of methyl violet gives very satisfactory results it was assumed that a mordant such as anilin oil was a necessary factor in making a good Gram stain. Aqueous solutions of methyl violet are coming into general use in Europe. That this has not occurred in America is due to the difficulty of obtaining methyl violet 6b, as recommended by Jensen and to the fact that the American dyes have given unsatisfactory results. With the production of better domestic dye stuffs or improved methods of using the present products we believe that the favored primary stain will eventually be an aqueous solution which will be stable over a long period of time. This stability, combined with ease of preparation, are factors in its favor.

The present paper describes the results of experiments to compare aqueous solutions of various domestic dye stuffs and their value as substitutes for the anilin gentian violet solution in the Gram staining method.<sup>2</sup> The dyes used gave variable, and in most cases such unsatisfactory results that the experiments were extended to cover an analysis of the different factors determining the Gram reaction in the hope of modifying the staining method in such a way that satisfactory results could be obtained with more of the domestic dye products. The experiments included the determination of the effect of heat and of acid and alkali added to the primary stain on the slide, the effect of

<sup>2</sup> This work was begun by Mrs. Pearl M. Smith and continued by the writer.

washing between the different solutions, the effect of heat on the iodine solution, a comparison of different decolorizers and the effect of water on decolorization. Certain of the experiments have a bearing on the nature of the Gram reaction and the characteristics of the Gram precipitate.

*Organisms used.* Unless otherwise stated the smears used in these experiments were made from pure cultures of *Staphylococcus aureus*; *Bacterium typhosum*; *Neisseria catarrhalis* and *Neisseria gonorrhoeae*; grown for approximately twenty-four hours on peptic digest agar slants to which 33 per cent hydrocele fluid had been added or Loeffler's blood serum tubes.

*Films.* The films were made in tap water or physiological salt solution. A few preliminary experiments convinced us that no difference resulted from the making of the films in distilled water, tap water or salt solution. It was assumed that if a difference did result this would be a factor that would have to be taken into consideration in staining pus and body fluids. Mounting the films in an acid or an alkali does influence the results as will be described later.

#### DYES USED IN PRIMARY STAINING SOLUTION

One per cent aqueous solutions of six different samples of methyl violet, three of gentian violet and one of crystal violet were used and compared. The dye was added to the distilled water, shaken thoroughly, allowed to stand several hours and filtered as used. Such solutions remain stable for a considerable time. Some of our solutions were kept two months and showed no deterioration, in fact there seemed to be some improvement with age.

*Results obtained.* According to the method of Jensen, satisfactory results can be obtained with aqueous solutions of methyl violet 6b, only when using a strong iodine solution<sup>3</sup> and absolute alcohol as the decolorizer. With our dyes we compared the results obtained with the following decolorizers, 95 per cent alcohol, 100 per cent alcohol, 100 per cent acetone and acetone

<sup>3</sup> One gram of iodine, 2 grams of potassium iodide, 100 cc. of distilled water.

and ether (equal parts), otherwise following the method of Jensen except that safranin was used as a counter stain.

Briefly, Jensen's method is as follows: Air dry, fix with mild heat, cool before flooding with 0.5 per cent methyl violet solution for fifteen to thirty seconds; rinse off methyl violet with iodine solution, flood with fresh iodine solution for thirty to sixty seconds, drain off iodine solution and wash with absolute alcohol until stain ceases to come out of film; counter stain for fifteen to thirty seconds with neutral red made up as follows: 1 gram neutral red, 2 cc. glacial acetic acid, 1000 cc. distilled water.

With 95 per cent alcohol as the decolorizer only one of our dyes gave satisfactory results. Using absolute alcohol four of the dyes, three of the methyl violets and the crystal violet, gave good differentiation. Better results were obtained with acetone or acetone and ether than with absolute alcohol. These results show that all American methyl violet dyes do not give satisfactory results when used according to the method of Jensen. Of the dyes used one of the methyl violet dyes gave distinctly superior results and another distinctly inferior results. The crystal violet dye gave better results than some of the methyl violet and any of the gentian violet dyes used.

The dyes were found to vary in the amount of precipitate formed upon the addition of the iodine solution and the rapidity with which this precipitate went into solution in the decolorizer. The dye giving the poorest results produced a heavy precipitate which went into solution slowly. This required longer exposure to the decolorizer which partially accounts for the poor results. One of the essentials of a good dye should be that the precipitate formed with the iodine solution go into solution in the decolorizer very rapidly.

An attempt to modify the method of Jensen so that satisfactory differentiation can be determined with a larger percentage of American dye products was successful and is given in detail at the end of the article.<sup>4</sup> We will discuss here only certain

<sup>4</sup> The methyl violet dye giving the best results, i.e., resisting decolorization the longest, was a sample furnished by the Will Corporation of Rochester, New York, and submitted to us as their methyl violet No. 3. With the method of

parts of this technique which are deemed worthy of special attention.

EFFECT OF SODIUM BICARBONATE WHEN ADDED TO THE PRIMARY STAIN

The addition of a few drops of a strong solution of sodium bicarbonate to the dye on the slide improves the intensity of the stain in the Gram positive organisms. A few drops of 10 per cent lactic acid produces the opposite result. No attempt was made to determine to what these changes are due. We are possibly dealing with changes in osmotic pressure and subsequent concentration of the dye in the cell or with a change in the size of the molecule of the iodine-dye precipitate, or simply with a heavier precipitate. The sodium bicarbonate tends to precipitate the dye but the acid does not. The sodium bicarbonate and lactic acid do not produce a permanent change in the cell as is readily shown by exposure of the film to either one of the solutions and then changing the reaction by the addition of the other solution. A film so treated will stain as though the first solution had not been used (Burke, 1921).

The effect of the sodium bicarbonate is shown in staining films made from old cultures of Gram positive organisms. Such films stained by the ordinary methods show many organisms which are Gram negative and some which are Gram amphophile or Gram positive. Similar films stained with sodium bicarbonate added to the violet dye will show a larger percentage of Gram positive organisms. Apparently some of the Gram amphophile organisms have absorbed or retained a larger amount of the dye and appear Gram positive. Sodium bicarbonate does not tend to make a naturally Gram negative organism Gram positive. These facts suggest the possibility, not heretofore recognized, that the loss of the Gram positiveness of organisms in old cultures is not entirely due, as formerly assumed, to autolysis and altera-

staining described in this article satisfactory results were obtained with a crystal violet and methyl violet furnished by the National Stain and Reagent Company of Norwood, Ohio; three methyl violet and a gentian violet furnished by the Will Corporation of Rochester, New York; and a methyl violet and gentian violet furnished by the Harmer Laboratories of Philadelphia.

tion of the cell wall but may be due in part to the presence of acid.

The application of these facts in the study of slow growing organisms is obvious. Its value in the examination of pus and body fluids is also evident and has been discussed in a separate paper (Burke, 1921).

The sodium bicarbonate solution may be omitted from the Gram stain if the best dyes are used. We have found that 3 to 8 drops of a 5 per cent solution of sodium bicarbonate solution is usually sufficient to insure good results. If too much sodium bicarbonate solution is added a heavy precipitate forms and there is an almost complete separation of the dye from the water. This should be avoided. A film forms where a drop of strong sodium bicarbonate hits the dye. This disappears as the dye and sodium bicarbonate are thoroughly mixed.

Sodium bicarbonate should not be added to the stock solution of the stain as there results a rapid breaking down of the solution. Whether the sodium bicarbonate is just as effective when added to the iodine solution was not determined.

#### EFFECT OF WATER ON DECOLORIZATION

The presence of water on the slide to which the decolorizer is added has a marked effect on the rate and extent of decolorization. The rate and degree of decolorization in the presence of water depends to some extent, according to the physical conception of the reaction, upon the action of the water on the cell wall of the Gram positive organisms and upon the fact that the precipitate formed by the dye and the iodine goes into solution in the decolorizer more rapidly if kept moist than when allowed to dry. It follows that in making a stain it is advisable to remove as much water as possible from the slide without allowing the dye precipitate to become dry before adding the decolorizer. Also that after decolorization of the Gram negative organisms is complete water or an aqueous counter stain should not be added to the slide until the decolorizer has evaporated or been removed. The addition of water to the decolorizer decreases its power to take up the dye precipitate into solution and increases its rate of decolorization of the Gram positive organisms. This

is shown by the fact that eighty per cent alcohol decolorizes the Gram negative organisms more slowly and the Gram positive organisms more rapidly than 100 per cent alcohol. If the alcohol is diluted sufficiently both types of organisms decolorize at the same rate. With the addition of more than 50 per cent water the Gram negative organisms do not decolorize over night.

The explanation of these facts upon a physical basis is that (1) the precipitate is more soluble in alcohol or acetone than in water and (2) the water alters the cell wall of the Gram positive organism or reduces the size of the molecules of the dye precipitate so that the dye is more easily washed out by the decolorizer. With the addition of sufficient water to the decolorizer the Gram positive organisms or the molecules of the precipitate are so altered that the dye comes out of the Gram positive organisms as readily as out of the Gram negative organisms. Conversely the elimination of water from the decolorizer and from the cell results in the Gram positive organisms retaining the dye more tenaciously than the Gram negative organisms.

Unfortunately prolonged exposure to a water free decolorizer will remove the dye from the Gram positive organisms and thorough drying of the precipitate delays its solution in the decolorizer. It follows that if we dry the film too thoroughly before adding the decolorizer the rate at which the dye precipitate goes into solution may be decreased to such an extent that before the dye can be washed from the slide and the Gram negative organisms it may, but does not always, begin to come out of the Gram positive organisms. It is not all washed out of Gram positive organisms by acetone in 12 hours. By taking advantage of these facts it is possible by careful blotting of the film before adding the decolorizer to increase the Gram positiveness of the Gram positive organisms without affecting the rate at which the dye precipitate goes into solution in the decolorizer and the decolorization of the Gram negative organisms. Successful staining with some dyes depends upon the skill with which the water is removed from the film. This constitutes perhaps the most critical step in the process of staining by Gram's method. Success with the poorer American dyes depends upon one's ability to properly gauge the effect of the blotting upon the



decolorization of the Gram positive organism and the skilful control of the decolorizer. With the best dyes it is not necessary to pay special attention to this process although the excess water should always be removed by blotting.

There are then, in so far as water is concerned, three facts which should be understood, controlled and utilized in the making of a Gram stain: 1, Water added to the decolorizer increases its power of decolorizing the Gram positive organisms; 2, Water added to the decolorizer slows down the rate at which the dye-iodine precipitate is taken into solution; 3, A dye-iodine precipitate goes into solution before drying much more rapidly than after drying.

Since a small amount of water must be left in the film and the addition of water to the decolorizer affects the results it is inadvisable to use the decolorizer more than once as is sometimes done when decolorization is brought about by placing slides in Coplin jars. Also the decolorizer takes up but a small amount of the precipitate and quickly becomes saturated and then ceases to decolorize the Gram negative organism.

#### EFFECT OF THE IODINE SOLUTION

The addition of the iodine solution causes a heavy precipitation of the dye. This precipitate is insoluble in water, but readily soluble in alcohol or acetone and has no staining affinity for cells. It is washed out of the Gram negative organisms more rapidly than out of the Gram positive organisms. It is washed out of the Gram negative organisms more rapidly and from the Gram positive organisms less rapidly than the unprecipitated dye. According to the physical conception of the Gram reaction, the molecules of the Gram precipitate are of such a size that in solution in alcohol or acetone and in the absence of water they do not as readily pass through the limiting membranes of Gram positive as of Gram negative organisms. Neide (quoted by Benians, 1912) states that the potency of the Gram reaction depends largely on the strength of the iodine solution and on the length of the period of application. By using a solution containing double the amount of iodine present

in "Lugol's" solution or increasing the period of exposure to "Lugol's" solution he was able to make some Gram negative organisms retain the violet dye. He also claimed that heating the iodine solution on the slide tends to make the Gram negative organisms retain the stain. Benians studied the effect of steaming the iodine solution for five minutes and found that after this treatment of the *Bacterium coli* organisms either intact or crushed resisted decolorization with 100 per cent alcohol for five minutes. He assumes that the heat causes a chemical change in the dye-iodine precipitate in the presence of the bacterial cell substance and that the precipitate ceases to be soluble in alcohol.

If increasing the exposure to, or increasing the strength of, or steaming, the iodine solution causes the Gram negative organisms to retain the dye then these are factors to be controlled in making a Gram stain. However, in our own experiments we were unable to see that increasing the period of application to two hours or doubling the strength of the iodine solution (2-4-100) had any effect on the decolorization of Gram negative organisms. Likewise the steaming of the iodine solution on the slide for five minutes did not effect the decolorization of typhoid organisms. Films so treated decolorized as rapidly as when exposed to the iodine solution for one minute. If, however, the film became dry during the process the Gram negative organisms retained the dye much longer. In our experiments acetone was used as the decolorizer. The former workers used absolute alcohol which may account for the differences in results.

Our experiments convince us that if acetone or acetone and ether is used as the decolorizer and the film is not allowed to dry steaming or prolonged exposure to the iodine solution will not materially affect the decolorization of the Gram negative organism.

#### VALUE OF DIFFERENT DECOLORIZERS

*Alcohol 95 per cent.* Commercial 95 per cent alcohol is used in many laboratories as the decolorizing solution. It gives satisfactory results with some of the better dyes but it can not be used with the poorer dyes. As we have already shown the

addition of water to the decolorizer slows down the rate of the decolorization of the Gram negative and increases the rate of decolorization of the Gram positive organisms. Seventy-five per cent alcohol decolorizes the Gram positive almost as rapidly as the Gram negative organisms. Fifty per cent alcohol decolorizes both types of organism at about the same rate.

*Alcohol absolute.* Absolute alcohol gives better results than 95 per cent alcohol as there is a greater margin of time between the decolorization of the Gram negative and Gram positive organisms. The high cost and difficulty of obtaining absolute alcohol militate against its use.

*Alcohol and acetone.* The addition of acetone to the alcohol increases the rate of decolorization of the Gram negative organisms and slows down the rate of decolorization of the Gram positive organisms. Therefore a decolorizing solution of alcohol and acetone gives better results than absolute alcohol.

*Acetone.* Acetone decolorizes the debris on the slide and the Gram negative organisms from 5 to 10 times as fast as absolute alcohol and the Gram positive organisms much more slowly. Acetone does not completely decolorize the Gram positive organisms if they have been stained with a good dye, in 15 hours. With one of the dyes used by us absolute alcohol decolorized *Staphylococcus aureus* as much in fifteen minutes as acetone did over night. The addition of water to the acetone has the same effect on the decolorization of organisms as the addition of water to alcohol. Eighty per cent acetone gave as good results as 95 per cent alcohol.

*Acetone and ether.*<sup>5</sup> With 100 per cent acetone the decolorization of Gram negative organisms is almost instantaneous. If desired this rapid decolorization can be slowed down by the addition of ether to the acetone. One part of ether to 1-3 parts of acetone serves as a very good decolorizer. As ether costs about the same as acetone there is little or no economic advan-

<sup>5</sup> It is a common laboratory procedure to use a mixture of alcohol and acetone as the decolorizing solution. Lyon (1920) recommends the use of acetone alone as the decolorizer. We have found that a mixture of acetone and ether is just as satisfactory as acetone.

tage in adding ether to the acetone. Short exposure of the Gram positive organisms to the ether does not affect their Gram positiveness.

#### ADVANTAGES OF ACETONE AS A DECOLORIZER

1. Reduces time required for the decolorizing process.
2. Gives a greater time period between decolorization of Gram negative and Gram positive organisms.
3. Is cheaper and more readily obtained than absolute alcohol.
4. Gives better results with the poorer dyes.
5. Makes a cleaner slide as the dye is more thoroughly extracted from the debris and clusters of bacteria.
6. Gives better results as it decolorizes the Gram positive organisms more slowly than alcohol.
7. Makes possible the use of a stronger dye or a mordant such as phenol because any heavy precipitate on the slide is quickly washed away.

Alcohol has no advantage over acetone as a decolorizer in the Gram process of staining. When these facts become known we believe acetone will come into more general use as a decolorizer. As has been emphasized by Lyon (1920) acetone can take the place of alcohol in the preparation of pathological sections and in the cleaning and drying of pipettes and other glassware.

#### *Counter stain*

The choice of a counter stain and the strength of the solution to be used should be determined by a number of factors. With the better violet dyes the choice of a counter stain is relatively unimportant but with the poorer dyes the choice and control of the counter stain largely determines the excellence of the result. Some of the counter stains in general use have a greater tendency than others to mask the violet dye. One has the choice of either, first, shortening the decolorization with the possibility of leaving a trace of the violet dye in the Gram negative organisms and depending upon a strong counter stain or long exposure to cover over any violet dye remaining in the Gram negative organisms or, second, continuing the decolorization until the Gram negative

organisms are thoroughly decolorized, with the possibility of reducing the brilliancy of the dye in the Gram positive organisms and relying upon a weak counter stain not to mask the dye in the Gram positive organisms. The degree of contrast is the same in both cases but I prefer the former method with a strong counter stain like Safranin O which brings out the Gram negative organisms very distinctly and stains them a color which contrasts more sharply with the blackish purple of the Gram positive organisms than that of some other counter stains. I find it easier to make a decision and study the morphology if both types of organisms are heavily stained with distinct colors than if both are more weakly stained with distinct colors or if one type is intensely and the other very faintly stained.

#### XYLOL

Clearing the stained film in xylol or turpentine improves the definition, thereby making it easier to separate the Gram positive from the Gram negative organisms and to study the morphology. It is of particular value in the examination of pus and mixed cultures. By clearing the organism in this manner instead of depending upon the immersion oil one can more readily determine whether the density of color in some of the questionable organisms is due to a mixture of the violet dye with the counter stain, or to a masking of the violet dye by the counter stain, or to an excessive concentration of the counter stain.

#### GRAM REACTION

The phenomenon of the Gram reaction has been explained upon both a chemical and a physical basis. The chemical explanation of the reaction is that the dye, iodine and protein of the cell of the Gram positive organisms form a comparatively insoluble compound. The physical explanation is based upon the assumption, supported by some convincing experiments, that the phenomenon depends upon the nature of the cell membrane and the size of the molecules in the dye iodine precipitate, the molecules in solution in the decolorizer being unable to

pass readily through the cell membrane of the Gram positive organisms owing to the size of the pores. It does not come within the scope of this paper to give a critical analysis of the facts bearing on the above theories. For further discussion the reader is referred to a recent paper by Benians (1920) in which the evidence in support of a physical explanation of the Gram reaction is clearly presented.

We wish to describe here the results of attempts to apply to practical staining methods certain of the conceptions presented in Dr. Benians' article. The results obtained have some bearing on the questions involved and may stimulate others to further research along similar lines.

Benians divides bacteria into three groups as regards the Gram reaction: (1) Gram positive organisms into which the dye penetrates and from which the dye-iodine precipitate is not readily washed out by the decolorizer; (2) Gram negative organisms like the gonococcus into which the dye penetrates but from which the dye iodine precipitate is rapidly washed out by the decolorizer; (3) Gram negative organism of the coliform type into which the dye probably does not penetrate and under certain conditions is not even absorbed into the surface of the cell and which are therefore readily decolorized.

According to the physical conception of the Gram reaction the cell membrane of the Gram positive organism does not allow the passage of the compound dye-iodine molecule when in solution in the decolorizer. If the dye-iodine precipitate in solution in the decolorizer can not pass out of the cell due to the character of the cell wall then we are justified in assuming that under similar conditions the dye-iodine precipitate can not pass into the Gram positive cells. We can also reasonably assume that since the dye-iodine precipitate readily passes outward through the wall of Gram negative organisms like the gonococcus it will just as readily pass inward through the cell wall in so far as physical conditions operate. It follows then that if we expose films of staphylococcus and gonococcus to an alcoholic solution of the dye-iodine precipitate the dye-iodine compound should penetrate the gonococci but not the staphylococci. Also since 75 per cent

alcohol decolorizes the Gram positive organisms very rapidly a solution of the Gram precipitate in 75 per cent alcohol should stain the Gram positive organisms almost as rapidly as the gonococcus like organisms. If this dye-iodine compound could be forced to remain in the gonococci we could stain the films with a weak counter stain which would stain the staphylococci and we would have the phenomenon of a reversed Gram stain. The practical application of such a staining method in the examination of pus for gonococci is obvious.

Unfortunately the addition of iodine to the dye saturates its affinities so that an alcohol or acetone solution of the dye-iodine precipitate does not stain cells. By simply exposing films of organisms to the solution we can not determine whether the molecules of the precipitate have penetrated into the gonococci and not into the staphylococci. The staining power of the solution can be materially increased by the addition of alkali but we do not know what affect this has on the size of the molecules. If the molecules are reduced in size they should enter the Gram positive as well as the Gram negative organisms. If the size of the molecular groups is not altered by the addition of the alkali then the gonococci should be penetrated and stained and the staphylococci not penetrated and not stained or only the surface stained. Exposure to a counter stain or weak decolorizer and counter stain should stain the staphylococci and not the gonococci.

#### ATTEMPT TO REVERSE THE GRAM PHENOMENON

*Experiment 1.* Films of pure cultures of *Staphylococcus aureus*, *Neisseria catarrhalis* and *Bacterium typhosum* were made on a slide in physiological salt solution, air dried and fixed by heat. A sufficient amount of iodine solution was added to a quantity of an aqueous solution of methyl violet to cause a maximum precipitation, the precipitate washed to remove excess iodine and dried. The films were flooded with a saturated alcoholic solution of this precipitate. A few drops of a strong mixture of sodium bicarbonate, sodium phosphate and sodium hydroxide were added to the dye on the slide. After an exposure

of 10 minutes or more the slide was dipped in water to remove free dye and lightly counter stained with aqueous Safranin O. Upon examination the staphylococci were Gram negative and the other two types of organisms weakly Gram positive.

This experiment suggests that the dye-iodine precipitate in solution in alcohol with the addition of alkali more readily penetrates the Gram negative than the Gram positive organisms used. The difference noted apparently is not due to a more rapid decolorization of the staphylococci by the water. If the films are examined after washing and before exposure to the Safranin solution the three types of organisms appear to be equally well stained. Since the counter stain more quickly stains the staphylococci than the other two organisms we assume that the staphylococci are less heavily stained by the dye-iodine precipitate or are only surface stained rather than that the Safranin has a greater affinity for the staphylococci.

Staining by this method, as controlled at the present time is entirely inadequate from a practical point of view for distinguishing staphylococci from gonococci in mixed infections. The results obtained are not uniform and the degree of differentiation is not sufficient.

#### EXPERIMENTS TO DETERMINE WHETHER THE PRIMARY STAIN PENETRATES THE CELL WALL OF TYPHOID LIKE ORGANISMS

Benians' conception of two types of Gram negative organisms as described above is based upon two experiments as follows:

1. If gonococci and *Bacterium coli*, unfixed by heat, are shaken up in weak solution of methyl violet (1 to 40,000) and then centrifuged, the gonococci are thrown down well colored and the dye is cleared out of the solution while the *Bacterium coli* are thrown down uncolored leaving the whole of the dye in solution. If the *Bacterium coli* organisms were boiled or killed at 65° for thirty minutes they absorbed the dye and were thrown down well colored. The *Bacterium coli* organisms exposed to 60°C. for thirty minutes did not absorb the dye. If the unfixed bacilli were suspended in strong solutions they became deeply colored.

2. When films of *Bacterium coli* organisms and *Bacterium coli*



organisms ground in a mortar were placed on the same slide, fixed by heat in the usual way, treated for two minutes with 0.5 aqueous solution of methyl violet and then decolorized with 95 per cent alcohol it was found that the dye was held much more strongly by the ground up debris of the organism than by the intact. According to Benians "This seems to provide almost certain evidence that the dye had never really permeated the intact bacilli, to get into their substance, as it had been able to get into the substance of the bacilli when broken up. The dyes were therefore only absorbed to the exterior of these intact organisms."

We suggest here that the difference in rate of decolorization between the intact organisms and the amorphous material may have been due to a greater saturation and more rapid drying of the amorphous material rather than to an entire lack of penetration of the dye into the interior of the intact cells.

It seemed important to us, from a purely practical consideration of the subject, to determine whether all the members of the large coli-typhoid-dysentery group of organisms resisted penetration of the dye as had been demonstrated for *Bacterium coli* and to such an extent as to permit of differential staining between these organisms and the gonococcus like organisms. Our experiments were based on three assumptions: (1) That if the cell wall of these organisms resisted penetration by the dye it should resist decolorization of the cell if the dye could be gotten into the cell without altering the cell wall and the decolorizer did not affect the cell wall; (2) That if the organisms were stained with methyl violet, then stained with a dye such as Safranin which slowly covered over the methyl violet and then cleared in xylol the cells should not show a violet center and a Safranin periphery unless the violet had penetrated to the interior of the cells; (3) That if the organisms were stained with methyl violet, partially decolorized with acetone and counter stained with Safranin the center of the cells should not appear violet and the margin of the cell Safranin unless the violet had penetrated to the center of the cells.

*Experiment 1.* Films of *Bacterium typhosum*, *Staphylococcus aureus* and *Neisseria catarrhalis* were air dried and stained over night in a 0.5 per cent aqueous solution of methyl violet, then exposed to the decolorizing action of water, weak alcohol, 100 per cent alcohol, acetone, and chloroform. In all cases the typhoid organisms decolorized as rapidly or more rapidly than the other organisms.

*Experiment 2.* The experiment was repeated with carbol-fuchsin as the staining solution; similar results were obtained. Increasing the period of exposure over the usual two minutes period, increased the time required to bring about decolorization. Apparently the increased exposure brought about a greater concentration or greater penetration of the dye in the cell. There is of course the possibility of a chemical change resulting from the long exposure.

*Experiment 3.* The above experiments were repeated but with the application of an iodine solution in the usual manner. The typhoid and catarrhalis organisms decolorized at about the same rate and much more rapidly than the staphylococci when exposed to acetone.

From these experiments we conclude that after the dye penetrates typhoid organisms the cell wall offers no greater resistance to the removal of the dye than the cell wall of *Neisseria catarrhalis* and *Staphylococcus*. We have not demonstrated that the dye penetrated the cells but since a similar exposure will result in the staining of acid fast organisms we assume that the dye did penetrate. The following experiments favor this assumption.

*Experiment 4.* Films of *Bacterium typhosum*, *Neisseria catarrhalis* and *Staphylococcus aureus* were air dried, stained for two to three minutes in a 0.5 per cent aqueous solution of methyl violet, washed in water to remove excess stain, stained lightly with 2 per cent aqueous Safranin O, washed and cleared in xylol for ten minutes. Upon examination the staphylococci almost uniformly showed a safranin colored border with a dark center. The safranin had penetrated part way into the cells and masked the violet dye. The *Neisseria catarrhalis* organism resembled the staphylococci with the exception that the Safranin had not

penetrated so deeply. The typhoid organism showed less penetration of the Safranin than the staphylococci, in this respect resembling *Neisseria catarrhalis*, but an occasional organism showed a very definite violet center with a Safranin margin. The Safranin masked the violet dye in the staphylococci more rapidly than in the other two organisms.

*Experiment 5.* The experiment was repeated with films fixed by heat in the usual manner. The results were similar to that obtained with the unfixed films.

*Experiment 6.* The experiment was repeated with unfixed films with the exception that the smears were partially decolorized by a brief exposure to acetone and ether (equal parts) before being stained with the Safranin. Upon examination many of the typhoid organisms showed a very definite purple center surrounded by a Safranin margin. Some of the typhoid organisms showed irregularly spaced blackish granules in the center somewhat resembling a string of cocci and surrounded by a Safranin colored margin. Other of the typhoid organisms appeared uniformly Safranin colored. As far as the eye could determine the violet dye had penetrated to the center of many if not all of the typhoid organisms.

We conclude from these experiments that in ordinary Gram staining the violet dye penetrates through the cell wall of *Bacterium typhosum*. Our experiments failed to demonstrate that the cell membrane of typhoid like organisms offers much if any greater resistance to the dyes than the cell wall of *Neisseria catarrhalis*. The use of weaker dyes might bring out differences not evident when stronger dyes are used. The solution of methyl violet used by us was of the same strength as used by Benians in one of his experiments with *Bacterium coli* in which he apparently demonstrated that the dye did not penetrate to the center of the cell.<sup>6</sup>

<sup>6</sup> With the Gram stain used by us the typhoid organisms decolorized slightly more rapidly than the *Neisseria catarrhalis* organisms but as the latter were distinctly larger the difference in rate of decolorization may have been due to the difference in size.

These experiments were discontinued because they did not give promise of demonstrating a method by which we could stain the colon-typhoid group of organisms differently from the gonococcus like organisms.

#### A MODIFIED GRAM STAIN

The following method of making a Gram stain with aqueous solutions of dyes and without the addition of mordants to the stock solution of the primary stain gives better results than any other method known to us. With this method satisfactory differentiation between Gram positive and Gram negative organisms can be obtained with many of the domestic gentian violet, methyl violet and crystal violet dyes. With some of the better dye products certain of the steps in the process can be omitted and others modified but with the poorer dyes strict attention to all of the details given is advisable.

1. Air dry thinly spread film and fix with least amount of heat necessary to kill the organisms and fix them to the slide (A).
2. Flood smear with a 1 per cent aqueous solution of the dye to be used. Mix with the dye on the slide 3 to 8 drops of a 5 per cent solution of sodium bicarbonate, allow to stand two to 3 minutes (B).
3. Flush off the excess stain with the iodine solution<sup>7</sup> and cover with fresh iodine solution and let stand one minute or longer (C).
4. Wash in water as long as described and blot off all free water until surface of film is practically free of water, but do not allow the film to become dry (D).
5. Decolorize with acetone or acetone and ether (1 part ether to 1 to 3 parts acetone) until decolorizer flows from slide practically uncolored. This usually requires less than ten seconds (E).
6. Blot dry. The slide quickly dries without blotting (F).
7. Counter stain for five to ten seconds or longer if desired with a 2 per cent aqueous solution of Safranin O (G).
8. Wash off excess stain by short exposure to water, blot and dry (H).

<sup>7</sup> One gram iodine, 2 grams potassium iodide, 100 cc. distilled water.

Immerse in xylol or turpentine for several minutes or until clear. Examine.

If the first attempt at staining a smear does not give satisfactory results it is advisable to wash off the oil with xylol, wash off the xylol with acetone and restain. It has been our experience that restaining smears gives better results than the original attempt.

*A.* The film can be made in either distilled or tap water or physiological salt solution.

*B.* Some workers recommend cooling the slide before flooding with the dye. With some dyes steaming seems to improve the result or shorten the required period of exposure. Passing the slide through the flame until steaming begins and allowing to stand the two minutes is sufficient. Steaming does not cause the Gram negative organism to resist decolorization. Anilino-gentian-violet can be used in place of the aqueous solution if desired. Allowing the stain to dry around the edge makes a dirty slide but does not affect the Gram reaction. The strength of the solution of the dye and the period of exposure can vary somewhat without affecting the result. A saturated instead of a 5 per cent solution of sodium bicarbonate may be used.

*C.* The excess stain can be blotted off or washed off by a brief exposure to water. The exposure to the water should be as brief as possible as water tends to reduce the amount of dye in the cells. Washing with water has the advantage of giving cleaner slides and effecting a saving of the iodine solution and the decolorizer.

*D.* The iodine solution can be blotted from the slide but this has the disadvantage of leaving a small amount of iodine on the slide and with the volatilization following the addition of acetone there is some irritation of the exposed mucous membrane of the worker. If for any reason one can not complete the staining process after reaching this stage it is advisable to place the slide in water until the staining can be completed.

*E.* The decolorizer should be placed upon the slide, allowed to stand for a few seconds and drained off. Then fresh decolorizer allowed to flow over the surface of the slide until it drops off

clear. Proper control of this process will reduce the amount of decolorizer used to a minimum. Placing the decolorizer in a Coplin jar and dipping the slide up and down in it will not give satisfactory results.

*F.* Drying after the decolorizer is essential as the aqueous counter stain mixing with the decolorizer has an effect on the Gram positive organisms. The smear can now be examined for Gram positive organisms. The oil can then be washed off with xylene, the slide dried and the counter stain applied.

*G.* Various counter stains can be used but I prefer Safranin O, or neutral red as recommended by Jensen. With dyes giving a poor Gram reaction it is necessary to reduce the counter staining to a minimum.

*H.* Washing should be sufficient to remove the dye from the surface of the organisms, flushing for a few seconds will suffice.

#### CONCLUSIONS

1. Steaming the iodine solution on the slide or increasing the period of exposure does not cause the Gram negative organism to resist decolorization.

2. Drying the film after exposure to the iodine solution greatly delays decolorization of the Gram negative organisms.

3. There are two critical steps in the Gram staining method; (a) the removal of the water after the iodine solution and (b) the decolorization. The control of these steps determines to a large extent the amount of differentiation between the Gram positive and Gram negative organisms.

4. The addition of water to the decolorizer, either on the slide or in the bottle, retards the decolorization of the Gram negative organisms and increases the rate of decolorization of the Gram positive organisms.

5. Acetone is superior to absolute alcohol as a decolorizer. It decolorizes the Gram negative organisms and debris on the slide much more rapidly and the Gram positive organisms more slowly than absolute alcohol.

6. Mounting the films in distilled water, tap water or physiological salt solution does not affect the staining reaction. Mount-

ing the films in sodium bicarbonate or lactic acid greatly affects the result.

7. The addition of sodium bicarbonate results in a greater concentration of the methyl violet dye being present in the Gram positive organisms after decolorization and lactic acid brings about the opposite result. The failure of Gram positive organisms in old cultures and in smears from the genital-urinary tract to retain the violet dye may be due in part to the presence of certain acids. These facts suggest the possibility of enhancing the value of gentian violet in selective media and improving dye therapy by the addition of an alkali.

8. All of the colon-typhoid group of organisms do not differ from the gonococcus-catarhalis group of organisms in their resistance to the penetration of the methyl violet dye used in the Gram stain.

Results of an attempt to reverse the Gram reaction are described.

There is described a modified Gram stain which, without the use of mordants or alcohol, gives very good differentiation when using many of the American dye products.

#### RECOMMENDATIONS

1. That the Committee on Pure Culture Study of the Society of Bacteriologists instead of attempting to select any one Gram method to be used as the standard method in the study and description of pure cultures designate the control organisms to be used in checking up the stain. Any Gram method which would give sharp differentiation between the two control organisms could be considered a satisfactory Gram stain to be used in the study of pure cultures. The selection of the control organisms will require considerable study as the organisms selected should have the same size and morphology when grown under identical conditions and should require a first class stain to bring out sharp differentiation. The custom of using staphylococcus and typhoid as control organisms is not satisfactory. The control organisms selected could be sent out to the different

laboratories. We believe this method will bring about more uniform results than the selection of any standard Gram method.

2. That the dye manufacturers attempt to improve the dyes used in the Gram stain by the addition of alkali which will not affect the stability of aqueous solutions of the dye.

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## DISINFECTION STUDIES<sup>1</sup>

### THE EFFECTS OF TEMPERATURE AND HYDROGEN ION CON- CENTRATION UPON THE VIABILITY OF BACT. COLI AND BACT. TYPHOSUM IN WATER

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Exposure to extremes of temperature and of hydrogen ion concentration produces an accelerated death rate of bacteria. These are typical conditions of ordinary disinfecting procedures. Temperatures and hydrogen ion concentrations in the zones between those which are favorable and those which are distinctly lethal may be expected to produce an adaptation in the organisms or a comparatively slow death rate, knowledge of which may reveal the conduct of bacteria under moderately unfavorable conditions in nature, and add to our understanding of certain phases of disinfection.

The object of this study was to investigate the effects of variations in moderate temperature and moderate hydrogen ion concentrations upon the death rate in water and dilute buffer solutions. The experimental method imposed sub-lethal conditions upon the bacteria in contradistinction to the accelerated death induced by ordinary disinfecting procedures.

It has been found in unbuffered media like distilled water or tap water that the death rates vary coincidentally with apparently unimportant shifts in hydrogen ion concentration. When the pH factor is controlled by means of M/500 buffers, the death rates are stabilized so that comparisons become possible. The

<sup>1</sup> Published by permission of the Surgeon General. Thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Yale University.

pH zone of tolerance, or minimum death rate, of *Bact. typhosum* lies between 5.0 and 6.4; that for *Bact. coli* is wider, and centered near absolute neutrality. The effect of sub-lethal factors is to make a period of induction apparent before death proceeds at a logarithmic rate.

The phenomena of growth, maintenance and death of bacteria are fundamentally important to bacteriology, theoretical and applied; and there are conceivably numerous factors that may influence them. Omitting from consideration in this discussion the obviously large part played by the food supply, we recognize certain predominating physical and physico-chemical factors, chief among which are temperature and the concentration of hydrogen ions. In bacterial death, and in disinfection, which may be regarded as a special phase of bacterial death, their rôle may indeed become paramount.

Temperature plays its important part in the life and death processes of bacteria by controlling the active agencies involved. Referring to the death processes Clark and Lubs (1917) have said: “. . . . in cellular destruction temperature is to be considered as an accelerating *condition* . . . . among the active agents concerned the concentration of hydrogen ions may be of great significance.” This opinion is amply supported by experimental evidence reaching back to the days of Pasteur.

As an aid to the study of certain phases of biological processes, the effect of temperature has been widely used and has yielded facts of importance. It has been found empirically that temperature augments physical phenomena in arithmetical progression and chemical phenomena in geometric ratio. As a rule the temperature coefficient of velocity of most chemical reactions for a rise of ten degrees is 2 or more; and for physical phenomena it is nearer 1.

Snyder (1908, 1911) cites numerous examples of these two types of temperature coefficient and shows that in most physiological processes the temperature coefficient of chemical reaction velocity applies. Loeb (1908) ingeniously accounts for the much richer animal and plant life of Arctic waters by reasoning that since a temperature decrease of ten degrees increases via-

bility 1000 times, and of twenty degrees, one million times, while the rate of development is reduced one-third to one-ninth, it therefore follows that at 0°C. many more successive generations may exist simultaneously than at 10° or 20°C. Madsen and Streng (1910) found that the conservation of agglutinins was affected in a like manner; and Kanitz (1915) in his monograph on the subject collects many of the data for convenient comparison.

The rôle of temperature in the growth of bacteria has been recognized from the very beginning. Its effects are found alike in those life phases in a bacterial culture characterized by Buchanan (1918) as the logarithmic growth phase, the maximum stationary phase, and the logarithmic death phase. The present experiments extend the application to the period of accelerated death.

Ward (1895) by painstaking bacterioscopic studies worked out a curve showing the relation between the rate of growth of *B. ramosus* and the temperature of its environment. Barber (1908), doing the same thing with *Bact. coli*, found growth at 30°C. increased two to three times over that at 20°C., and Lane-Clayton (1909) confirmed this observation. Slator (1919) demonstrated the same relationship to hold for the growth and maintenance of yeasts.

Houston (1914) showed that as the temperature was decreased the viability of *Bact. coli* and *Bact. typhosum* in water was increased. The crude experiments of Konradi (1904) showed a similar relationship for staphylococci and other organisms; and Livingston (1921) finds this true for hemolytic streptococci. The magnitude of the temperature effect observed by these authors may only be inferred, however, for their data are mainly qualitative in nature. Paul, Birstein and Reuss (1910) give quantitative data upon the viability of staphylococci and find a temperature coefficient of 2 to 3 for a 10° rise. In the process of disinfection we have numerous examples and a mass of exact data that show the general application of this rule. In this connection we need only mention the classic researches of Paul and Krönig (1896), Madsen and Nyman (1907), Paul (1909) and of Chick (1908, 1910, 1912). In the heat sterilization of bac-

terial spores, Bigelow and Esty (1920) find that the time of sterilization is increased ten times for a  $10^\circ$  reduction in sterilizing temperature. The Committee on Standard Methods of Examining Disinfectants of the American Public Health Association in its last report (1918), has made recommendations for the inclusion of the temperature coefficient as one of the three necessary items in the characterization of disinfectants.

As has been stated above, the relation between temperature and the speed of chemical reactions is as yet upon an empirical basis. van't Hoff (1896) attempted a derivation from thermodynamic considerations but obtained no definite solution. Arrhenius (1889) assumed that reacting substances occur in two tautomeric forms, "active" and "passive," and that a certain quantity of heat is involved in activation. He suggests a relation of the form:

$$v_1 = v_0 \cdot e^{\frac{q}{R} \left( \frac{1}{T_1} - \frac{1}{T_0} \right)}$$

for the velocities of reaction,  $v_0$  and  $v_1$ , at absolute temperatures  $T_0$  and  $T_1$ , the quantity  $q$  being the constant of activation. Numerous empirical formulae of the same general form have been suggested from time to time; and that of Snyder (1911) is one that he applied to physiological processes. Tolman (1921) discusses the views that have been held and points to the lack of a real fundamental explanation of the temperature effect in monomolecular reactions. Dushman (1921) and Lewis and McKeown (1921) offer theories of chemical reactivity which appear to be based upon fundamental considerations and include the effect of temperature.

There is an ever-increasing literature upon the effects of hydrogen ion concentration<sup>2</sup> in many biological processes, as a glance at the references quoted by Clark (1920) will show. In the field of bacteriology its importance as a controlling factor is definitely established. This control is exercised in many subtle and unexpected ways—upon the activity of specific

<sup>2</sup> In this work, the Sorensen symbol pH is used synonymously for the hydrogen ion concentration.

enzymes, upon toxin production, upon the dissociation of essential foodstuffs and upon the state of aggregation of cellular protoplasm. Our present meager knowledge permits us usually to see only the end result in growth, metabolism or death.

Cohen and Clark (1919) found that the acid limit for the growth of *Lactobacillus bulgaricus* is not identical with that for fermentation, thus suggesting a possible distinction between bacterial growth and metabolism. In the experiments to be reported in this work there is a hint that we might extend this distinction to include bacterial death.

These factors, temperature and hydrogen ion concentration, have been utilized in the present investigation to study some of the characteristics of that phase of disinfection produced by mild factors, which for want of a better term we have called sub-lethal.

Beginning with the first systematic studies by Koch (1881), who was later followed by Paul and Krönig (1896), Krönig and Paul (1897), Madsen and Nyman (1907), and culminating in the achievements of Chick (1908, 1910, 1912), there has been evolved a theory of disinfection based upon well known principles of physical chemistry. This may be summarized by the dictum of Phelps (1911): "The rate of dying, whether under the influence of heat, cold or chemical poison, is unfaillingly found to follow the logarithmic curve of the velocity law, if the temperature be constant." Lee and Gilbert (1918) come to a like conclusion after a critical investigation.

On the other hand there are some like Reichel (1909), Loeb and Northrop (1917), Brooks (1918), Peters (1920) and Smith (1921) who urge that the logarithmic process is only an apparent one, and that careful study of the intimate nature of the disinfection curve will show it to be dependent upon the individual resistance of the organisms. Brooks supports the contention of Loeb and Northrop that bacteria are distributed according to resistance upon a probability curve and that bacteria of low resistance die off first. Since the logarithmic law takes cognizance of the number of organisms without regard to the distribution of resistance among them, the operation of this law among bacteria would, so they claim, be an unnatural process.

To throw some light upon this controversy and at the same time aid in an understanding of the mechanics of the disinfection process, it seemed that a study based upon the following considerations might prove useful. Most previous studies of disinfection were based upon results of the application of an intense disinfecting agent, like heat, heavy metals, acids, alkalis, etc. As a result, the response of the organisms was prompt and the period of their adjustment to the new conditions was so small as to be overlooked. Only in exceptional cases, as the one cited by Chick, where old cultures of *Staphylococcus aureus* were used, was the early period long enough to excite attention; but in that case the cultures used could not be considered as fairly homogeneous. If the conditions causing death of the organism could be toned down in intensity to a degree that might be termed sub-lethal, then there should be an opportunity to observe their adjustment from their manner of response.

The reduction of lethal intensity may be satisfactorily accomplished by maintenance of the organisms at sufficiently low temperatures on the one hand and by the control of the hydrogen ion concentration of their environment on the other.

#### EXPERIMENTAL

The series of experiments reported and discussed below was planned to give information on the following aspects of the behavior of bacteria as represented by the colon-typhoid organisms.

1. The response of bacteria to the sub-lethal factors (in contradistinction to disinfection as ordinarily understood) of starvation and moderate intensities of hydrogen ion concentration.
2. The effect of moderate temperatures upon the rate of this response.
3. The analysis of the behavior in the light of the physico-chemical concept of the disinfection process.

A statistical method of approaching a solution of these questions appears at present to be the only satisfactory one; and briefly stated, the method of study pursued was to expose *Bact. coli* or *Bact. typhosum* to a given solution and follow periodically

the numbers of survivors capable of forming colonies on nutrient agar.

### *Materials and apparatus*

Close attention was paid to obtaining uniformity of conditions throughout the work. Constant temperatures (10°, 20°, and 30°C.) were secured in an electrically heated and controlled, well-stirred air thermostat, with fluctuations probably not greater than 0.5°C. In the experiments conducted at 0°C. the bottles were maintained at the temperature of melting ice.

The plating materials were of the usual kind, consisting of graduated pipettes, petri dishes, dilution bottles and tubes, and nutrient agar.

The glassware was cleaned in the usual manner and sterilized by dry heat at 160°C. for five hours or longer. The dilution bottles were of about 250 cc. capacity, and were filled from an automatic burette with 100 cc. of distilled water. The dilution tubes were filled with 10 cc. of water. Sterilization in the autoclave brought the contents down on the average to 99 cc. and 9 cc. respectively.

The procedure in making distilled water the suspension-fluid or the diluting fluid is at variance with the customary practice of using so-called physiological salt solution or mixtures of this solution with nutrient broth. This variation was made advisedly because preliminary tests had shown quite conclusively that for our purposes distilled water was a satisfactory neutral fluid (cf. Zeug, 1920).

The nutrient agar for plating was made by a uniform method at various times from a single lot of Difco proteose peptone and Liebig's beef extract. It contained per litre:

	<i>grams</i>
Peptone.....	10
Liebig's beef extract.....	3
Shred agar.....	20

The reaction of the medium was adjusted colorimetrically to pH 7.0. Occasional tests of the reaction of the nutrient agar at the time it was actually used for plating showed the hydrogen ion concentration to be uniformly between pH 6.7 and 6.9.

*The distilled water* of the laboratory, from a gas-heated Stokes automatic still, was used in all cases except where otherwise mentioned. In experiments requiring water of exceptional purity the latter was prepared by double distillation out of acid and alkali in Pyrex glass, with precautions for the exclusion of carbon dioxide.

*The tap water* used was Potomac River water which had presumably been treated with alum and filtered through sand. Ficker (1898) has shown that an "oligodynamic" property is readily acquired by water which is allowed to remain in contact for any long time with the metallic fixtures in ordinary plumbing. To prevent this occurrence in our water, the latter was allowed to run freely for several hours before being used for experiment. During filtration, a by-pass provided a constant flow of fresh tap water under a head of one meter.

*The buffer solutions* were prepared with the precautions described by Clark (1920). The only divergence made here was to dilute these buffers to a concentration of M/500. The pH values of these dilutions were then determined colorimetrically. Tests were made to determine the minimal amount of buffer necessary to maintain constant hydrogen ion concentrations under the conditions of these experiments; and it was found that the M/500 concentration of buffer answered this purpose most satisfactorily.

*The bottles* in which the bacterial viability was studied were of approximately 1000 cc. capacity, of ordinary glass and with ground stoppers. Before use, they were thoroughly cleansed with fresh chromic acid cleaning mixture, rinsed, steamed in an Arnold sterilizer for several hours, and well rinsed with distilled water.

Except for the experiments with double-distilled water, in which cases the containers received an internal coating of purified paraffin, no attempt was made to prevent possible solution of the glass in the contained water. This procedure was followed because in preliminary experiments a comparison of the viability curves from water in Jena glass containers and in the above softer glass bottles showed no appreciable differences. When



precautions were taken during manipulation to avoid unnecessary and excessive heating, the amount of glass constituents dissolved apparently played no significant part in the experiments as conducted.

Paraffin melting at 55°C. was boiled with several changes of distilled water, stirred frequently and finally skimmed off into clean sterile bottles. The latter were then placed in a hot air sterilizer for several hours and later allowed to cool while a uniform coat was deposited internally.

*The organisms* selected for experimentation were two members of the colon-typhoid group, *Bact. coli* and *Bact. typhosum*. These were chosen both because they are well suited for this type of study; and because they have been extensively studied along related lines so that results obtained here may be readily compared and may add to the continuity of our knowledge.

Both cultures had been grown for a long time upon artificial media but this does not constitute a defect in the present experimental plan. The undoubted acquisition of higher resistance to external influences altered somewhat the degree of the mortality rate by accentuating and magnifying the retardation during the early phases of the process, precisely the condition desired.

*Bact. coli.* This organism was isolated from a polluted stream in 1916. Its cultural and morphological characters are typical of the *Bact. coli-communis* (sucrose-negative) type. Winslow and Falk (1918, 1920) have utilized this strain in their studies on salt antagonism.

*Bact. typhosum.* This was a culture of the Rawlings strain (no. 608) obtained from the American Museum of Natural History, New York.

Stock cultures were preserved on nutrient agar at 10°C. At least five daily transplants on agar at 37° were made before a culture was used for experiment.

*The procedure in making the tests.* The requisite number of bottles were filled with buffer solution or water, allowing an air space of about 100 cc. and the whole sterilized at one time in the autoclave at 15 pounds pressure for ten minutes. Exceptions

to this procedure were made in the case of the double-distilled water and the filtered tap water experiments. The double-distilled water was freshly distilled directly into sterile paraffined bottles, and the filtered tap water was filtered through a Berkefeld (N) porcelain candle into sterile bottles. The bottles were then placed in the constant temperature box at 0°, 10°, 20° and 30°C., respectively. Duplicate bottles were maintained at each temperature, and kept there at least 12 hours to assume their required temperatures before the beginning of the experiment.

Cultures of the organisms to be studied were grown on agar slants at 37° for sixteen to eighteen hours. A platinum loopful of the growth was carefully removed and shaken into 9 cc. of sterile distilled water. Precaution was taken to remove only the bacterial growth and to avoid taking up any of the medium. A homogeneous suspension of the organisms was then obtained by thorough shaking. The tube was allowed to stand for several minutes to permit sedimentation of particles and the supernatant fluid was used to inoculate the bottles.

The bottles, immediately after being inoculated, were thoroughly shaken to distribute the organisms evenly throughout the volume, and samples were taken to determine the bacterial content by removing 1 cc. of the suspension and plating it in suitable decimal dilutions. Duplicate plates were made of each dilution and, usually, there were 3 or 4 dilutions and sometimes more, of a single sample.

After a suitable period of incubation, usually 48 hours at 37°C., the bacterial colonies on the plates were counted. For the organisms maintained at 0°C. it was necessary to incubate for a longer period to permit growth of colonies of adequate size. The plates were counted and the results of duplicate plates averaged and noted as the bacterial count for the particular moment that the sample was taken.

Bottles were removed from their respective temperature surroundings only for minimal intervals, not more than a minute or two, usually. Otherwise, they were maintained, protected from light, at their respective temperatures. Successive samples to determine the bacterial content were taken at regular

intervals, and at each such time the bottles were thoroughly shaken to secure an even distribution of the bacteria.

Samples were also taken regularly for the colorimetric determination of pH and for the analysis of the absorbed gases.

*The determination of absorbed gases* in the water was carried out as follows: 5 cc. of the sample were transferred quickly to a Van Slyke (1917) gasometric CO<sub>2</sub> apparatus. The contained gases were extracted, CO<sub>2</sub> determined by means of absorption with KOH, the oxygen determined by means of absorption with alkaline pyrogallol and the volume of the residue noted. Preliminary analyses of air and of air-saturated water showed the method to be satisfactory for this purpose.

By these procedures, a study was made of the viability of *Bact. typhosum* and *Bact. coli* at 0°, 10°, 20° and 30°C. in double-distilled water, in tap water and in dilute buffer solutions.

#### *The experimental results*

In early experiments, the mortality of the organisms was often variable, and could be attributed possibly to the presence of soluble constituents from the glass containers. It was first suspected that some salt effect perhaps might be responsible. The results of the first four experiments show clearly the marked effect upon the mortality which was coincident with apparently small variations in hydrogen ion concentration.

*Behavior in unbuffered solutions.* The result of Experiments 1 to 4 may be most conveniently considered under one head. In these experiments *Bact. typhosum* and *Bact. coli* were each exposed to distilled water and to tap water at 0°, 10°, 20° and 30°C. The course of events is graphically presented in the accompanying charts (figs. 1 to 4).

All the figures are plotted with time as abscissae and the logarithms of survivors as ordinates. The resulting curves show the actual rate of decline in numbers. That is, the slope of a curve (the graphic equivalent of the velocity coefficient,  $k$ ) at any point is indicative of the speed of disinfection and is strictly comparable with the slope of any other curve on the same chart. The experimental points are connected by straight lines without effort to draw "smoothed" curves through greatly

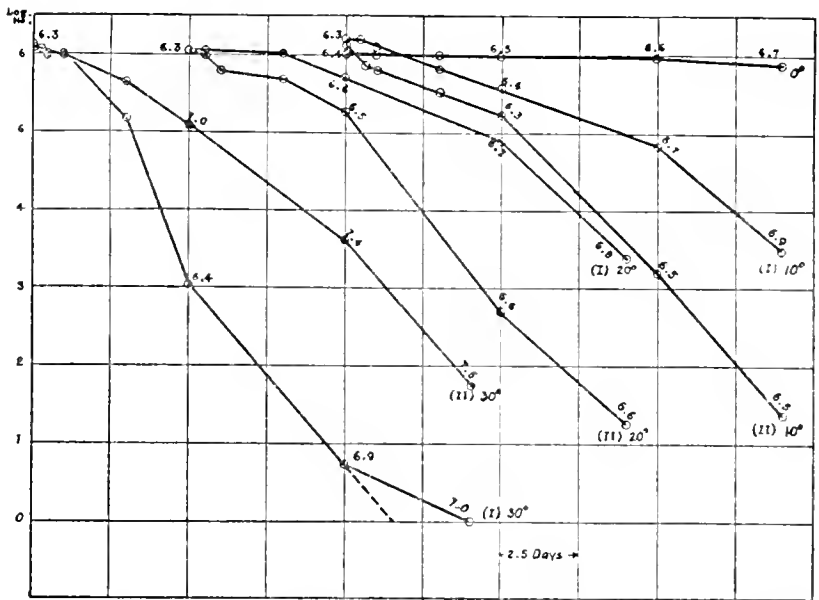


FIG. 1. EXPERIMENT 1. THE DEATH RATE OF BACT. TYPHOSUM IN DOUBLE-DISTILLED WATER AT 0°, 10° 20° 30°C.

Logarithms of numbers of survivors are plotted as ordinates against time intervals as abscissae. Duplicates at any temperature are marked (I) and (II). Observed pH values of the water are noted along the curves.

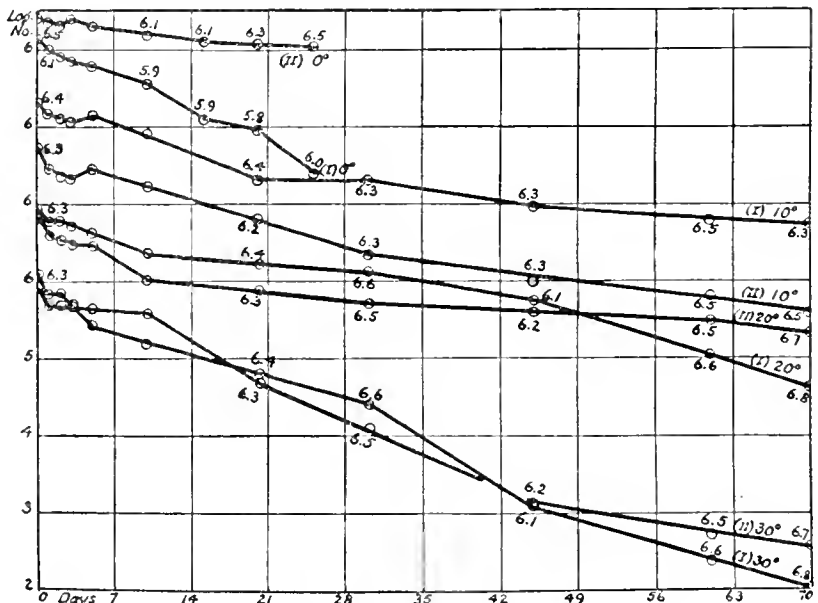


FIG. 2. EXPERIMENT 2. THE DEATH RATE OF BACT. COLI IN DOUBLE-DISTILLED WATER AT 0°, 10°, 20°, 30°C.

Duplicates at any temperature are marked: (I) and (II). Observed pH values of the water are noted along the curves.

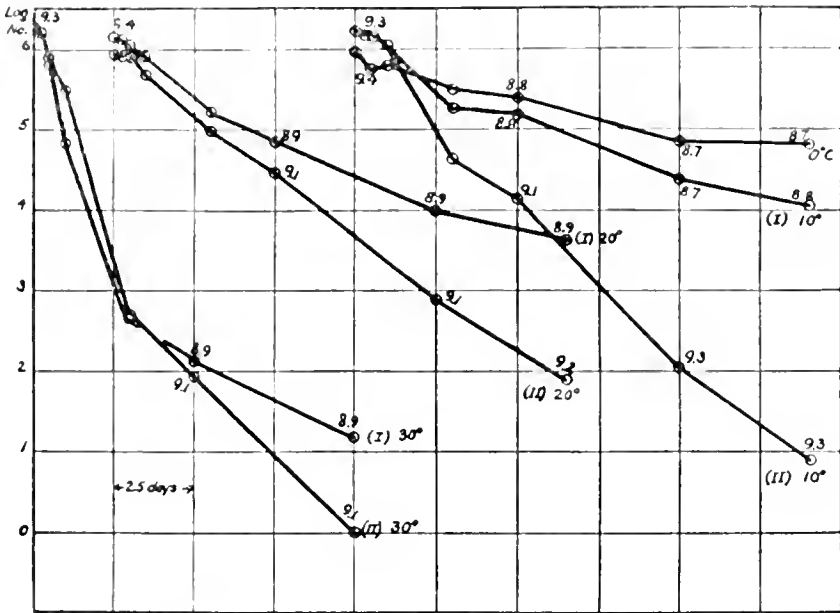


FIG. 3. EXPERIMENT 3. THE DEATH RATE OF BACT. TYPHOSUM IN AUTOCLAVED TAP WATER AT 0°, 10°, 20°, 30°C.

Duplicates at any temperature are marked: (I) and (II). Observed pH values of the water are noted along the curves.

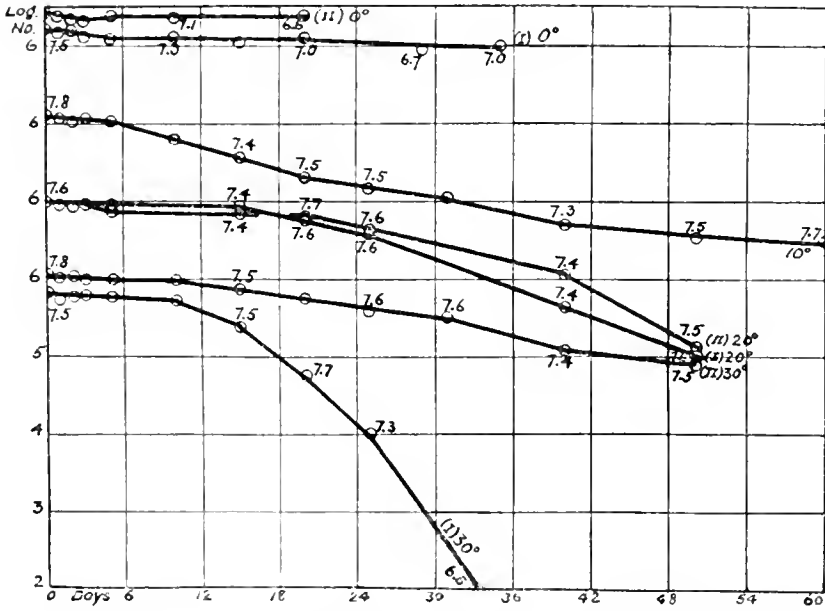


FIG. 4. EXPERIMENT 4. THE DEATH RATE OF BACT. COLI IN BERKEFELD-FILTERED TAP WATER AT 0°, 10°, 20°, 30°C.

Duplicates at any temperature are marked: (I) and (II). Observed pH values of the water are noted along the curves.

deviating points. It is true that the error in the experimental method employed is not inconsiderable and that a smooth curve might adequately represent the course of events occurring but there is also a danger of the eye being misled by an apparent regularity where none may exist.

The well-known higher resistance of *Bact. coli* is here again exemplified. As will be seen further on, this resistance is of utility in helping to throw light upon bacterial behavior during the early stages of disinfection.

Examination of figures 1 to 4 shows certain striking facts that are common alike to both organisms in unbuffered surroundings. The rate of decline in numbers does not always run parallel in duplicate bottles held at the same temperature. This perplexing result is consistently found in these four experiments and inspection at once shows that the divergence is the result of a chance distribution, some curves being generally parallel and others far apart. Of course, this divergence if present increases with the time elapsed, and depends upon the organism; for *Bact. typhosum* being a matter of days, and for *Bact. coli*, one of weeks.

These results were further substantiated six months later, when *Bact. typhosum* was studied in autoclaved tap water. This experiment, no. 12, was carried out in triplicate, and every precaution was taken to treat the samples alike. Yet in spite of these efforts, the mortality in each of the triplicate bottles followed a different course. (See table in Appendix.)

What could account for the divergence of duplicate bottles? They were handled alike as nearly as possible, and the bottles themselves, so far as we knew, were all of the same origin. The dissolved gases could not be responsible, for, as will be seen later, equilibrium with the air was attained within six hours from the beginning of the experiment; and furthermore, in experiment 4 filtered tap water, which presumably retained all of its dissolved gases, yielded the same result.

A possible clue is furnished by the pH values of the water determined at successive intervals. These values are noted along the curves on the charts, and it becomes evident that du-

plicate bottles giving parallel curves show but insignificant deviations in pH from each other. On the other hand, a wide divergence of the curves is coincident with appreciable fluctuations in pH.

These findings apply alike to double-distilled water and to Washington tap water. In the case of the distilled water, which contained a minimum of dissolved salts, the possible contaminants are the atmospheric gases of the laboratory which include, among others, ammonia and sulfur dioxide. The tap water contained in addition to the gases, about 163 parts per million of dissolved mineral matter. Both waters are poorly buffered, with their pH equilibria situated on the steep portion of a curve, so that a trace of strong base or acid will cause a relatively large change in the hydrogen ion concentration. This circumstance makes it difficult to estimate the pH accurately, for the hydrogen electrode cannot be used, and the addition of free indicators, which are themselves strong acids, may vitiate the results. We have, however, made a colorimetric estimation of the pH by utilizing the sodium salts of the Clark and Lubs indicators and comparing the results with those obtained with the free acids.

These pH deviations fall within the physiological range of our organisms, as will be shown later, and it could scarcely be urged, therefore, that these insignificant shifts were the cause of the large variations in mortality. It may however be that the slight pH changes observed were indicative of large and more profound local fluctuations in the unbuffered waters surrounding the bacteria; but it is worthless to pursue these thoughts further for they are purely speculative, and we are ignorant of all the forces operating in the present case. Yet the obvious inference occurs, that the control of the pH with buffers might stabilize a possible factor in the problem and lead to more consistent results. Later experiments are concerned with this aspect of the problem.

Our observations suggest that unbuffered solutions may not be depended upon to retain a constant and uniform hydrogen ion concentration for long, so that we must conclude that results of like experiments in the past may have been vitiated by this factor. Falk (1920) mentions the importance of pH in the study of salt effects upon the viability of bacteria.

It is evident from mere inspection of figures 1 to 4 that effects of temperature, whatever they may have been, were completely screened.

*The air-saturation of the suspension fluids*

Several considerations made it seem desirable to learn to what extent our water samples were saturated with air gases. The method of analysis used makes no claim to absolute accuracy, but gives a good idea of the actual condition present. Table 1 shows that distilled water, seven days old, was fully aerated at each temperature. To determine how soon saturation would

TABLE 1  
*Dissolved oxygen and carbon dioxide in distilled water held at different temperatures for seven days*

TEMPERATURE	VOLUMES PER CENT			
	Found		Calculated	
	O <sub>2</sub>	CO <sub>2</sub>	O <sub>2</sub>	CO <sub>2</sub>
0°	0.98	0.08	0.986	0.072
10°	0.80	0.06	0.796	0.047
20°	0.68	0.03	0.649	0.036
30°	0.53		0.526	

occur under the conditions of our experiments, the following experiment was carried out. Bottles containing 900 cc. distilled water and an air space of 100 cc. were autoclaved, allowed to cool while closely stoppered and then held at 0°, 10°, 20° and 30°C. respectively for twelve hours. The bottles were then thoroughly shaken, and samples removed for gas analysis as indicated in table 2. The results show that within four to six hours the waters were practically saturated with air. (CO<sub>2</sub> determinations are not given because the minute quantities present were within the limits of error of the method employed.)

These observations confirm occasional tests which showed that our bacteria were exposed to an air-saturated environment. As a consequence we may assume that the gas tensions were practically invariant at each temperature throughout these



experiments. That is, the decline in bacterial population at each temperature occurred under practically constant concentration of atmospheric gases. It is interesting to note, however, that at 0°, 10°, 20° and 30°C. the concentrations of oxygen occurred in the ratio of 986:796:649:526. This observation takes on a certain theoretical significance when considered in the light of the findings of Paul, Birstein and Reuss (1910) that dried staphylococci die off at a rate approximately proportional to the square root of the oxygen concentration. The experi-

TABLE 2  
*The reabsorption of atmospheric oxygen by autoclaved distilled water\**

WATER HELD AT	VOLUMES PER CENT OF OXYGEN				
	Hours after twelve-hour incubation				Calculated for air-saturated water
	0	2	4	6	
0°	0.54	0.88	0.97	0.97	0.986
10°	0.41	0.70	0.79	0.81	0.796
20°	0.25	0.58	0.64	0.66	0.649
30°	0.11	0.47	0.52	0.52	0.526

\* The water was autoclaved for ten minutes at 15 pounds, allowed to cool to room temperature and was held in an air thermostat for twelve hours. The water was well shaken before each successive sample was removed for analysis.

ments of Whipple and Mayer (1906) in this connection are interesting. They found that exclusion of oxygen from water containing colon and typhoid bacteria resulted in a rapid decrease in the bacterial population. The contradiction between these observers may perhaps be more apparent than real.

Jacobs (1920) has shown for tadpoles that CO<sub>2</sub> may produce a lethal effect which is accounted for by a specific penetrability. Koser and Skinner (1921) studying the viability of the colon-typhoid organisms found that carbonation of water causes a rapid diminution in numbers. Our experiments throw no light upon this phase of the problem. Carbon dioxide was present in our solutions in presumably constant, small amounts; and its effect should be present to the same degree in all experiments.

*The influence of soluble glass constituents*

Chemists from the time of Lavoisier have had to consider the question of the solubility of the glass vessels in their work. That a marked influence upon biological experiments may be exerted by soluble constituents from the container has been recognized now and then, but this aspect is yet too often neglected.

Some biological investigators like Beneke (1895) and Ficker (1898) have observed marked responses referable to glass constituents dissolved in the distilled water they used. Molisch (1895) tried to eliminate this source of difficulty by coating his vessels with paraffin. Houston (1914) attempted a comparison of containers of different materials upon the viability of bacteria and obtained some striking qualitative differences. Bigelow and Esty (1920), Esty and Cathcart (1921) and Fabian and Stull (1921) observe differences in heat sterilization referable in part to the solubility of the glass containers, and suggest that the result may be due to pH effects.

Under moderate conditions of temperature, the quantity of material dissolved by water from ordinary laboratory glassware in a short time is comparatively small;<sup>3</sup> and the most obvious results of such solution are the effect upon the pH of the water and the dissociation of other cations and anions. The results of the experiments reported here show that changes in pH of distilled water may often be quite considerable; and tap water, whether sterilized or filtered, has shown itself not very different in behavior from distilled water in this respect.

There are occasional references in the literature to a toxic action exerted by distilled water upon microbes. Laird (1919) found distilled water toxic to staphylococci, which fact he considers, as does Burgess (1920), to be due to plasmolysis and suggests "equilibrated" salt solutions as indifferent suspension media for bacteria. No observations of a similar nature seem to have been reported for bacteria of the colon-typhoid group. Our own experiments demonstrate the prolonged viability of these

<sup>3</sup> It should be noted that our glassware had been in use for some time and had become "aged" to some extent.

organisms in distilled water and tap water, and we must therefore conclude that in this bacterial species plasmolysis is not a very prominent phenomenon.

Winslow and Falk (1918) have demonstrated very interesting salt effects and antagonisms upon *Bact. coli* dying in water; and Holm and Sherman (1921) note similar effects upon growing bacteria. The suggestion of Zeug should be recalled in this connection. It is conceivable that such effects occurred in our experiments but were overshadowed by other more prominent factors. In the light of our experience with the activity of unbuffered suspension media upon bacterial death we must appreciate at once the great experimental difficulties involved in the study of pure salt effects, and the need for caution in their interpretation.

*The behavior of Bact. typhosum at different hydrogen ion concentrations*

The study of the mortality of bacteria at constant hydrogen ion concentration was made in M/500 solutions of Clark and Lubs' phthalate and phosphate buffers. It was considered desirable to keep the concentration of salt down to a minimum, and this dilution of the buffers was found to be the lowest that would maintain the pH unchanged under our conditions.

Experiment 5 (*Bact. typhosum* at pH 3.8, 5.0, 5.4, 6.4, 7.1, 7.6, 8.7 and 9.5) was performed to determine if constancy of pH would condition a uniformity in the results from duplicate bottles; and to learn the effect of pH at constant temperature (20°C.) upon the viability. Figure 5 shows that the effect of maintaining a constant pH throughout the experiment was to produce a high uniformity in the results from duplicate bottles.<sup>4</sup> As a consequence, duplicate curves, while determined in all cases, are omitted from the rest of the charts for the sake of clarity in presentation.

Reference to figure 5 shows that decline occurs at all hydrogen ion concentrations, and is least at pH 5.0 and 5.4. At pH 3.8,

<sup>4</sup>These are not selected specimens, but include all the tests made.

corresponding roughly to the acidity of N/10,000 HCl, the decline is most acute, and by contrast, at pH 5.0 (equivalent to about N/100,000 HCl), the rate of decline is the smallest. On

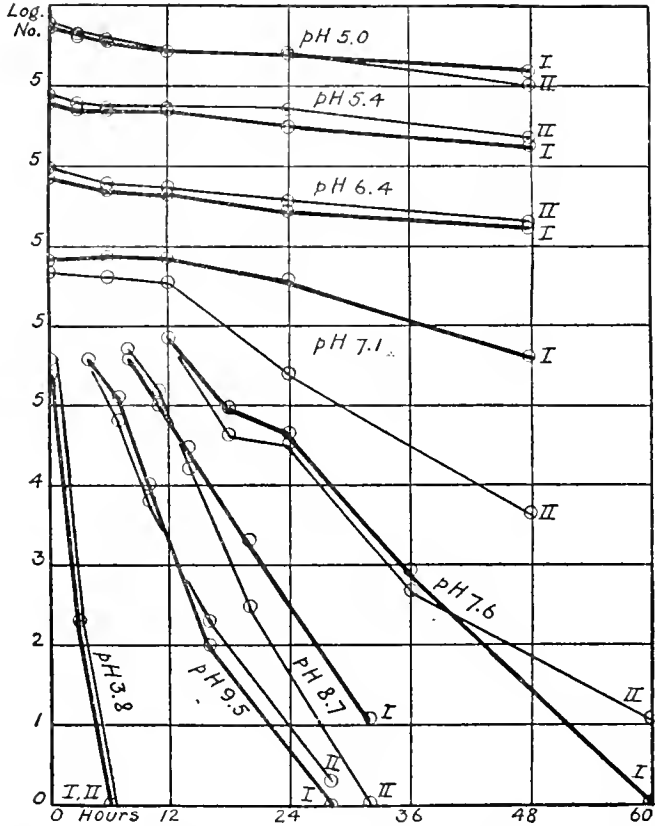


FIG. 5. EXPERIMENT 5. THE DEATH RATE OF BACT. TYPHOSUM IN M/500 BUFFERS AT 20°C.

The curves actually start from nearly the same origin, but have been spread apart to avoid confusion. Note the close parallelism between duplicates at almost all pH values.

the alkaline side, the acceleration in death rate is more gradual. Northrop (1920) studying the acid stability of pepsin found pH 5.0 to favor the greatest stability. He found for this enzyme, however, that increase of acidity did not destroy it so markedly as increase of alkalinity.

Our curves being derived under the same conditions are comparable and their relations may be designated numerically by their slopes, provided the curves are fairly straight lines, as in the present case. This is another way of stating that we are comparing *rates* of decline, or reaction velocities if we prefer to think in terms of the monomolecular reaction. The velocity constants thus derived are given in table 3.

This mode of expressing differences in disinfection has many advantages and has been recommended by Phelps (1911) as one of the logical procedures in the evaluation of disinfectants. Buchanan (1918), Bruett (1919) and Salter (1921) have utilized it in studies of heat sterilization and thermal death points.

TABLE 3

*Average velocity constants for the death of Bact. typhosum at different pH values at 20°C.*

	pH 3.8	pH 5.0	pH 5.4	pH 6.4	pH 7.1	pH 7.6	pH 8.7	pH 9.5
<i>k</i> .....	1.055	0.0134	0.0110	0.0138	0.0437	0.1100	0.2134	0.2855
*Relative <i>k</i> .....	95.5	1.2	1.0	1.5	4.8	10.0	22.4	31.4

\* Relative *k* represents the magnitudes of the various constants compared with that at pH 5.4 taken as unity.

The velocity constants we have thus derived show clearly that there is a zone between pH 5.0 and 6.4 in which *Bact. typhosum* declines in numbers very slowly. A small change in pH toward the more acid side of the zone produces an almost hundred-fold increase in the rapidity of death; while on the alkaline side a similar change in pH increases the death rate only four or five times. In brief, a small increase in the concentration of hydrogen ions at the acid end of the zone of tolerance produces a profound effect, whereas relatively large increases of hydroxyl ions produce a much smaller effect. This coincides with the findings of Cohen and Clark (1919) who called attention to a similar effect upon the *growth* of members of the colon group of bacteria.

This observation is of some importance theoretically, and may help to throw light upon the mechanism involved in the death of

bacteria. For instance, the optimum for growth of *Bact. typhosum* lies between the pH limits 6.2 and 7.2, though it will tolerate greater extremes. Yet here we encounter the significant fact that its optimum for maintenance under conditions of starvation lies between pH 5.0 and 6.4. It would, however, be unwise at present to dwell upon the possibilities in this direction.

Of interest in the same connection is the work by Shohl and Janney (1917) who established the pH limits for the growth of colon and typhoid bacteria in urine. Marsh (1918) found that *Bact. typhosum* is sensitive to the degree of acidity occurring in sour milk; and Beckwith (1920) reports that this organism is capable of surviving in rabbit bile *in vivo* even when the hydrogen ion concentration is depressed to pH 9.4.

It may be asked how far we are justified in concluding that the control of the pH with buffers conditions uniformity in results between duplicates. Striking graphic proof is given in Experiment 5 in which only one (that at pH 7.1) out of 8 tests failed in this respect. In experiments 6 to 13, the data for which are tabulated at the end of this paper, we find the large majority<sup>5</sup> of duplicates to run closely parallel. That there are a few exceptions is evident but even in these, the average divergence is very much less than in duplicates from deviating unbuffered solutions.

A further test of this fact was made six months later when *Bact. typhosum* was studied in triplicate samples buffered around pH 6.4. In this experiment, no. 13, the technique was especially careful, and the individual bacterial counts are the most accurate we have made, the probable error being below 5 per cent. If the resulting data be plotted against time, it will be found that each of the triplicate samples yields an almost identical curve. We have reason to believe that non-uniformity of results under these conditions is due mainly to laxity in technique.

<sup>5</sup> Of 33 such tests, about 4 may be considered to have shown any considerable divergence between duplicates.

*The behavior of Bact. typhosum and Bact. coli at pH 3.5 at different temperatures*

Since it became possible to eliminate variability in the results between duplicate bottles by means of control of pH, we could proceed to the study of the effect of temperature upon the death of these organisms.

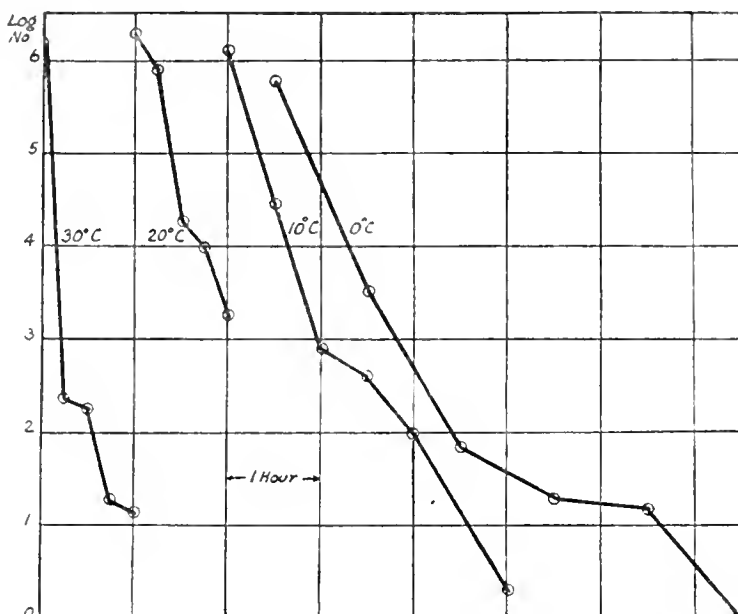


FIG. 6. EXPERIMENT 6. THE DEATH RATE OF BACT. TYPHOSUM IN M/500 PHTHALATE BUFFER, pH 3.5 at 0°, 10°, 20° 30°C.

Closely parallel duplicate curves are omitted

Experiments 6 and 7 were conducted at a comparatively high acidity (pH 3.5) in order to obtain a comparison of the relative rates of dying of these two organisms at 0°, 10°, 20° and 30°C. Such a comparison is not feasible at less intense acidities because of deviations of the disinfection curve from the simple logarithmic form, due to the predominance of certain factors referable to the specific peculiarities of each organism. This will be considered later.

Figures 6 and 7 present the curves of the death rates observed. These experiments are strictly comparable for they were done

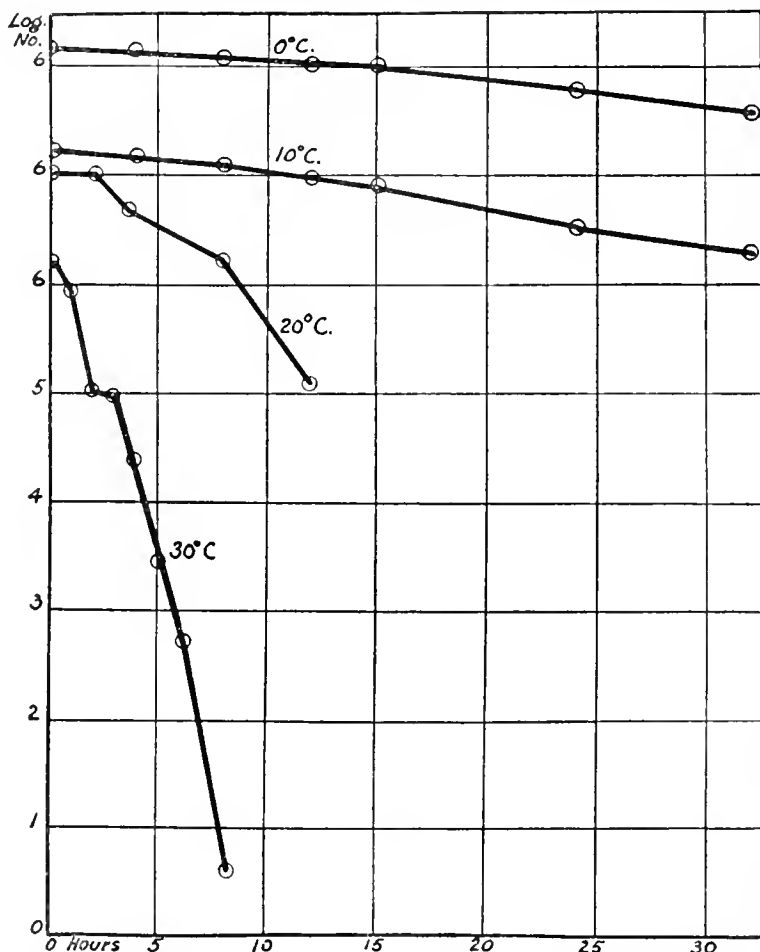


FIG. 7. EXPERIMENT 7. THE DEATH RATE OF BACT. COLI IN M/500 PHTHALATE BUFFER, pH 3.5, at 0°, 10°, 20°, 30°C.

Closely parallel duplicate curves are omitted

at the same time and with identical materials, and the marked difference in resistance of the two organisms is at once apparent. In the case of *Bact. typhosum* (fig. 6) the first portion of each



mortality curve is very nearly a straight line, but at the lower end there is a tendency to flatten out somewhat. In the case of *Bact. coli* (fig. 7) there is a suggestion of this tendency at the beginning of the curve. If we take the average velocity coefficients for comparison, the results may be arranged as in table 4.

We note in the case of *Bact. typhosum* that the increase in the death rate is fairly regular for 10° intervals, being 1.62, 1.53 and 1.77 from 0° to 30°C., while for coli the corresponding increases are 2.12, 4.36 and 3.76. With *Bact. coli* the effect of temperature is to increase the velocity for the 10° intervals from 0° to 30°C. in the ratio of 1 : 2 : 9 : 35 which corresponds closely to the exponential series: 2° : 2<sup>1</sup> : 2<sup>3</sup> : 2<sup>5</sup>. Such a relation is not evident in the behavior of *Bact. typhosum*.

TABLE 4

Average velocity coefficients for the death of *Bact. typhosum* and *Bact. coli* at pH 3.5 at different temperatures

TEMPERATURE	BACT. TYPHOSUM		BACT. COLI		$\frac{k_t}{k_c}$
	$k_t$	$Q_{10}$	$k_c$	$Q_{10}$	
0°	1.186	1.62	0.0176	2.12	67
10°	1.919	1.53	0.0373	4.36	51
20°	2.928	1.77	0.1654	3.76	18
30°	5.176		0.6214		8

$Q_{10}$  is the temperature coefficient for the indicated 10° interval.

If the ratio of velocity coefficients be considered a measure of the relative resistance of these organisms, we observe that at 0°C. *Bact. coli* is 67 times as resistant as the typhoid bacillus. As the temperature is increased by ten-degree intervals the relative resistance of *Bact. coli* decreases so that at 30°C. it is only eight times that of *Bact. typhosum*.

*The behavior of Bact. coli at different hydrogen ion concentrations and temperatures*

An inspection of the curves for the death of *Bact. coli* at pH 3.5 (fig. 7) shows that at 30°C. the curve is almost a straight line with a slight suggestion of flattening out at the beginning. At 20°C. this flattening out is more pronounced, and at 10°C. and 0°C., still more so. These deviations cannot be fully accounted for by the probable experimental error, though the process of

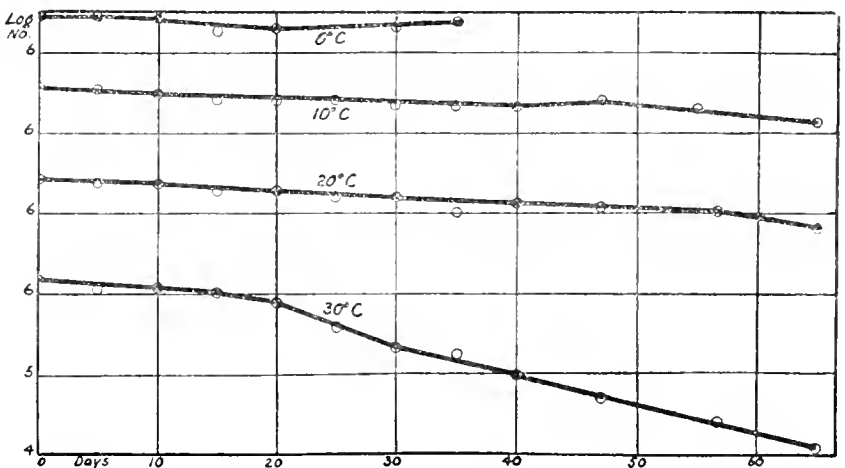


FIG. 8. EXPERIMENT 8. THE DEATH RATE OF BACT. COLI IN M/500 PHOSPHATE BUFFER OF pH 6.1 at 0°, 10°, 20°, 30°C.

Parallel duplicate curves are omitted

drawing 'smoothed' curves through the points may be made to wipe out this deviation. We have found, in experiments not detailed here, that at higher acidities these deviations from the straight-line logarithmic decline are absent so that the resulting curves resemble those found for *Bact. typhosum* in experiment 6.

If we now refer to figures 8, 9 and 10, it will be observed that these deviations are enhanced, especially for the lower temperatures. Such deviations simply mean that as time progresses

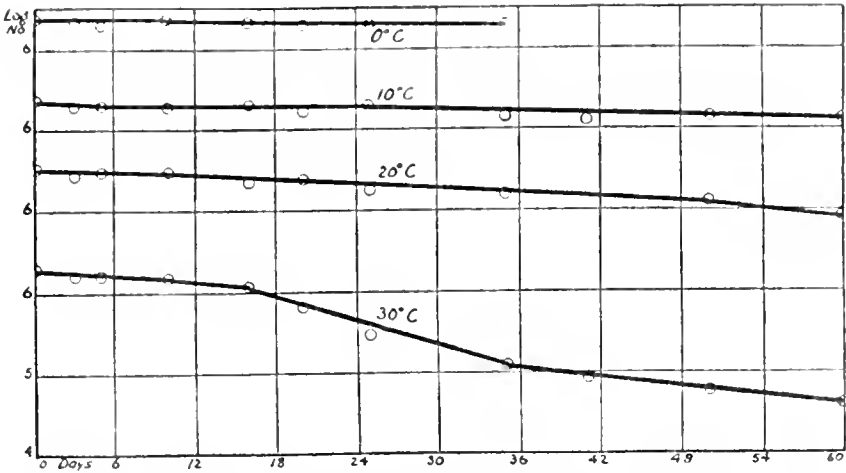


FIG. 9. EXPERIMENT 9. THE DEATH RATE OF BACT. COLI IN M/500 PHOSPHATE BUFFER OF pH 7.1 at 0°, 10°, 20°, 30°C.

Parallel duplicate curves are omitted

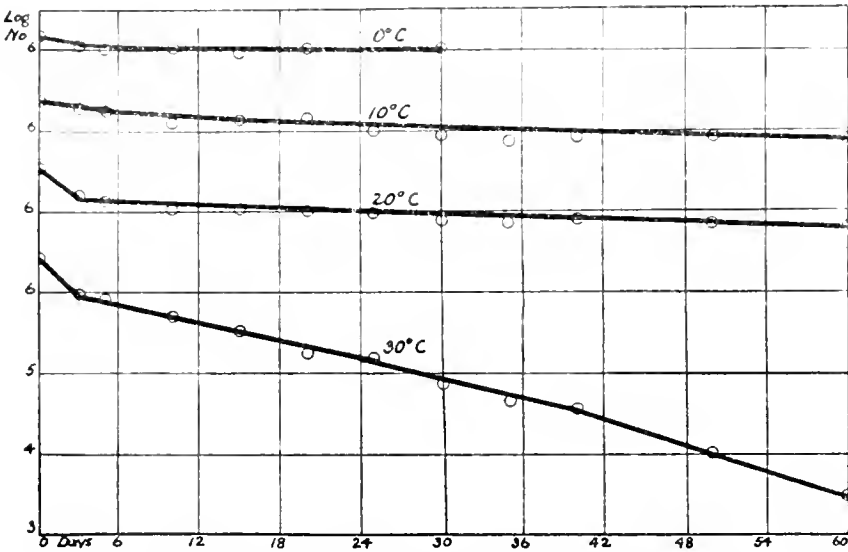


FIG. 10. EXPERIMENT 10. THE DEATH RATE OF BACT. COLI IN M/500 PHOSPHATE BUFFER OF pH 8.0 at 0°, 10°, 20°, 30°C.

The pH remained constant until the fortieth day, but was found to be 7.7 on the sixtieth.

the speed with which the bacteria perish increases to a maximum when the curve becomes a straight line. This phenomenon among bacteria finds its counterpart in certain chemical reactions which possess an induction period prior to reaction at the maximum rate. If the time necessary to reach maximum velocity in death rate is short then the calculation of an average velocity coefficient involves an insignificant error. This represents the mathematical equivalent of drawing a straight line as close as possible to the experimental points. If, however, this induction period is prolonged, then such a procedure may involve serious difficulties.

TABLE 5  
Average velocity coefficients of mortality of *Bact. coli* at different pH values and temperatures

TEMPERATURE	pH 3.5		pH 6.1		pH 7.1		pH 8.0	
	<i>k</i>	<i>Q</i> <sub>10</sub>	<i>k</i>	<i>Q</i> <sub>10</sub>	<i>k</i>	<i>Q</i> <sub>10</sub>	<i>k</i>	<i>Q</i> <sub>10</sub>
0°	0.0176		0.000206		0.000107		0.000260	
		2.12		1.53		1.62		1.22
10°	0.0373		0.000315		0.000174		0.000314	
		4.34		1.12		2.99		2.12
20°	0.1654		0.000353		0.000520		0.000666	
		3.76		4.56		2.29		3.00
30°	0.6214		0.001611		0.001170		0.001996	

An exact numerical measurement of the extent of this deviation is unfortunately not possible because of the limitations of the present experimental method. It may, however, be inferred that it is an inverse function of the temperature. Evidently at 30°C. it lasted about twenty days in experiments 8 and 9; and at 10° and 20°C. the end was not reached in sixty days. As stated above, a comparison of the average velocity coefficients is not justified as a logical procedure under the circumstances; but with this reservation we may consult table 5 to get an idea of the magnitudes involved.

This table indicates that the pH zone favoring the lowest destruction rate of *Bact. coli* is around absolute neutrality. It appears that this zone covers a greater range of hydrogen ion concentration than does that of *Bact. typhosum*.

We find in these experiments striking examples of that preliminary period in the disinfection process which we have referred to as the period of induction. The bacteria do not begin to die off at the maximum rate, but the mortality increases to the maximum gradually, depending upon the pH and the temperature. The lower the temperature and the less extreme the acidity, the greater is the duration of this period. We have succeeded in this case, apparently, in magnifying that early phase in disinfection which ordinarily is so small as to escape observation. Its significance will be discussed presently.

#### DISCUSSION

The probable error involved in the method of experimentation leaves much to be desired. Under the most nearly ideal conditions some of the results involve a probable error of only 2 to 3 per cent of the mean values indicated. In others, the probable error amounts to 10 per cent of the mean or more. When the bacterial numbers per unit volume are low, then the probable error increases greatly. These considerations led to the choice of an initial number of one million per cubic centimeter as involving the least manipulative error.

It might perhaps be well to express here a word of caution in regard to a too ready use of the calculated velocity coefficient,  $k$ , in the comparison of disinfections under varying conditions. This mode of expressing differences in disinfection has many advantages, provided its use implies no unwarranted assumptions. The general curve of disinfection, plotted logarithmically, is a straight line in the main, but at each end there are characteristic deflections. If these deflections are of small extent then an average velocity constant calculated for the whole of the disinfection period will involve no serious error. If, however, there is a lengthened period of induction before disinfection proceeds at the maximum rate, then obviously, such a basis for the calculation of the velocity constant would involve a large error. The same objection would hold for the condition in which the disinfection curve flattens out toward the end of the process. The latter consideration prompted Phelps (1911) to recommend the

determination of the velocity constant up to the point where the numbers of organisms have been reduced by 50 per cent. To avoid error from the former consideration as well,  $k$  should be determined for the middle part of the disinfection curve, say, between the points for 25 and 75 per cent reduction.

*The temperature coefficient ( $Q_{10}$ ) in bacterial viability*

We have already mentioned the broad empirical generalization that the temperature coefficient is about 1 for the acceleration of physical processes by a rise of  $10^\circ$  in temperature, and 2 or more for chemical processes. The rule is not a hard and fast one, and its basis is obscure. It is therefore important to recognize that the coefficient is at best a suggestive observation until we possess a better knowledge of the mechanism of the temperature effect.

Obviously, in strictly controlled experiments where only one reaction is allowed to take place, a temperature coefficient so derived is of some value. On the other hand, in biological experiments, the observed effect may be the resultant of an unknown number of independent as well as interdependent reactions, which may be both physical and chemical in nature. A temperature coefficient derived under such circumstances must be of doubtful significance. Changes of temperature may affect consecutive reactions in totally different ways. Mellor (1909) and Osterhout (1917) discuss this aspect of the problem and cite illuminating examples.

With these considerations in mind we may note that the temperature coefficients found in this investigation are in the main those to be ascribed to chemical reactions. This would indicate that somewhere in the series of consecutive processes ending in death, the slowest or limiting reaction velocity was that ascribable to the ordinary chemical reaction. Of more theoretical interest is the finding in experiment 7 that the death rates for *Bact. coli* increased for  $10^\circ$  intervals from  $0^\circ$  to  $30^\circ\text{C}$ . in an exponential ratio of the form:  $2^0 : 2^1 : 2^2 : 2^3 : 2^4$ , a regularity that seems more than accidental. Its interpretation is difficult for the present but we have here a suggestion for a possible method

of getting a closer view, perhaps, of one of the mechanisms involved in bacterial death. In this connection might be mentioned the contribution of Watson (1908) who showed that variation in the rate of disinfection due to change in concentration of the disinfectant could be expressed as an exponential function of the concentration.

Another apparent effect of increasing temperature is to decrease the period of induction prior to the logarithmic rate of death. The evidence is incomplete, but it would seem that the duration of induction varies in inverse proportion to some exponent of the temperature.

*The effect of pH upon bacterial viability*

We have seen that an uncontrolled hydrogen ion concentration may affect the death rate variably in unbuffered surroundings, so much so as to obscure the effects of wide ranges of temperature. When the pH is controlled by means of dilute buffer solutions the death rates become stabilized. Under the latter conditions there becomes evident a zone of pH in which the death rates of the bacteria are at a minimum. For *Bact. typhosum* this zone lies between pH 5.0 and pH 6.4 and for *Bact. coli* it is wider, with the optimum near absolute neutrality (pH 7.0). These zones may be regarded as optima for tolerance under moderate lethal conditions and when compared with the optima for growth show interesting relations, especially in the case of *Bact. typhosum*. The pH zone for optimum growth of this organism lies between 6.2 and 7.2, while here we have found that the zone for greatest tolerance lies between pH 5.0 and 6.4. Cohen and Clark (1919) showed for the colon-dysentery group that a slight increase in acidity beyond the optimum limit for growth caused a very large effect by preventing growth. The same phenomenon has been found in the present study of response to lethal conditions, where a slight increase in acidity caused a prompt change from maximum tolerance to high mortality.

In practical disinfection the intensity of attack, presumably is so great that such variations in tolerance as we have observed would hardly affect the bacterial death rates. Yet the possibility

remains that if a disinfectant be applied in a medium having a pH favorable to high bacterial tolerance, the result might be appreciably affected. It appears that this tolerance constitutes one element in the general condition termed "resistance." The foregoing observations furnish corroboration of the present well-established principle of determining disinfectant values under accurate control of the hydrogen ion concentration (Wright, 1917).

*The laws governing the disinfecting process*

We may now turn to a consideration of the process of disinfection and the bearing of our experiments upon the laws governing it. To treat the subject fairly and avoid the possibility of misunderstanding, we shall begin from certain fundamental concepts in physical chemistry. One of them is the assumption of the validity of the mass law. This states that at any moment, the velocity of a chemical reaction is dependent upon the relative masses of the reacting bodies as well as their nature.

If only one substance is undergoing change in a reaction then according to the mass law, the velocity of such change will depend upon the nature of the substance and its amount at any given moment, temperature and other conditions remaining constant. This statement of the law of monomolecular reactions is expressed concisely by the relation

$$V = C \cdot k \dots \dots \dots (1)$$

in which  $V$  represents the velocity of reaction,  $C$ , the amount of substance and  $k$ , a characteristic factor depending upon the nature of the substance undergoing change.

The velocity may be expressed by  $\frac{dx}{dt}$ , in which  $x$  represents

the amount of substance changed in the time  $t$ ; and if the original amount of the substance be designated by  $a$ , then  $a-x$  will represent the amount remaining after the time  $t$ . We may now write the above equation in the form



$$\frac{dx}{dt} = k(a - x) \dots \dots \dots (2)$$

This expression on integration becomes

$$k = \frac{1}{t} \log \frac{a}{a - x} \dots \dots \dots (3)$$

which is the familiar equation of the velocity of a monomolecular reaction and often spoken of as the logarithmic law.

It is perhaps well to emphasize that this formula represents the *course* of events taking place and makes no pretence of indicating the *mechanism* involved. It indicates the effect of active mass. Although the value of  $k$  will be greatly modified by change in the nature and intensities of the forces concerned, the essential *form* of the velocity curve will in no wise be altered since it is fixed by its inherent relation to the numbers of reacting molecules and by this factor only. The factor  $k$  may include, by its very definition, many influences. Therefore, to establish experimentally the applicability of the monomolecular law in any given case, all the influences must be kept constant while the effect of mass is being observed. For the present, our knowledge of the mechanism of a monomolecular reaction is nil. We may only guess that it involves complex electronic relations (cf. Tolman (1921) and Dushman (1921)). Note that  $k$  in the final expression happens to be designated as the velocity constant since time is inversely related to it. If  $\log(a - x)$  be plotted against time in the above equation, the resulting graph will be a straight line.

The above theoretical considerations have therefore led us to the deduction of a logarithmic equation that should hold good under the ideal conditions imposed. When we turn to actual experimental observations on monomolecular chemical reactions it is found that this logarithmic law holds good, according as we are able to maintain the ideal conditions. There may occur deflections from the true logarithmic rate at the beginning and the end of the reaction so that instead of a straight line plot of the results, we get a somewhat s-shaped curve. If conditions

are properly selected, the deflections may be reduced considerably. As a matter of experimental fact, they have never yet been completely eliminated (except possibly for radioactive substances).

These considerations are not held to affect the validity of the monomolecular law. They lead us rather to investigate the disturbing influences which, when discovered and eliminated, furnish further support to the law.

We now possess a fundamental concept of the origin of the monomolecular law as related to chemical reactions and may turn to the phenomena observed in disinfection. If the disinfection process be followed by noting the numbers of surviving bacteria at successive intervals we find that the rate at **which** disinfection proceeds is in general proportional to the number present. This proportionality is not absolutely true but generally so, and the graph representing the course of the process is more or less a straight line with deflections at both ends. Under appropriate conditions these deflections may be eliminated so that the curve becomes very nearly a straight line. The mortality process as observed by Chick and others, when strong disinfecting agents are used, has been shown conclusively to follow the logarithmic rate, or a slight modification of it.

It is needless to stress the self-evident analogy between the course followed by a monomolecular chemical reaction and the course of the mortality process of bacteria. They represent actual observations.

In this connection must be mentioned the statistical deduction of Yule (1910) based upon the theory of probability, that in a population exposed to a single lethal influence, the rate of death will follow a logarithmic course, and when there are a number of sub-lethal causes, the death rate will be a modification of the logarithmic one.

The above facts have been fully explained by Chick and it would seem superfluous to repeat them were it not for the criticisms of Chick's conclusions by Loeb and Northrop (1917), Brooks (1918) and Smith (1921). These authors urge that individual variations among bacteria may be distributed on a frequency curve such that the mortality rate will proceed *apparently* logarithmically.

Now it would of course be extravagant to say, since the course of disinfection is found to follow the monomolecular law, that therefore a single molecular species is concerned. We can only say that whatever the mechanisms are and whatever the number of consecutive reactions may be, they leave dominant the effect of bacterial concentration. We may say in other words that we are fortunate in having to deal with a phenomenon in which the effect of concentration can be found, and that we can formulate the course of the disinfection process in terms of an equation expressing the relation of concentration to the course of the process. We are, aware, however, of some of the factors that affect the course of the reaction. These in the case of chemical reactions, are enumerated and discussed by Mellor (1909). One of them, the effect of successive intermediate reactions, has been applied by Osterhout (1917) in an interesting manner to biological phenomena, and by Winslow and Falk (1920) to controvert the notion that disinfection is due to a distribution of variable resistances.

We have already indicated that even in the case of a simple chemical reaction known to involve only one molecular species, the mechanisms of disintegration are unknown, or at least the subject of dispute. The monomolecular law in such cases shows only the course of the reaction as it is related to the concentration of reacting bodies.

The analogy in the two cases should be plain. In neither case does the monomolecular law tell us anything about the mechanisms concerned. In both cases the monomolecular law formulates the relation between concentration and the course of the process.

If then we say that the course of disinfection is determined by the distribution of varying resistances we add nothing to the formulation of the experimental facts. We could just as well say that the course of a monomolecular chemical reaction is determined by the frequency distribution of resistance among the individual molecules.

In the criticism by Loeb and Northrop, by Brooks and by Smith use is made of the fact that at the beginning and end of the dis-

infection curve there are deviations from the monomolecular rate. This is emphasized as evidence that the monomolecular rate at the middle portion of the curve is more apparent than real. However, with cultures having presumably the same distribution of resistances we have found that the shape of the beginning and end of the curve may be modified at will by changing the exterior conditions.

Consequently, what force do these objections to Chick's theory possess? The chance distribution of variable resistances assumed for bacteria is paralleled by the chance distribution of variable energy quotas in the molecules of a substance. Furthermore, the logarithmic rate observed, whether in chemical phenomena or in disinfection, is actually a statistical resultant of like significance in both.<sup>6</sup> From such considerations we must conclude that the criticisms of the monomolecular theory of the disinfection process are of no fundamental force for they reduce to a matter of definitions only.

It appears that Chick's contribution has a larger significance than merely the application to a special phase of bacterial existence. Yule's statistical deduction, the experimental findings of Loeb and Northrop regarding the viability of *Drosophila*, those of Brooks regarding hemolysis of red blood cells and a number of others of like import, all indicate the operation of a general principle.

#### CONCLUSIONS

1. The mortality at constant temperature of bacteria in unbuffered media like distilled or tap water is variable and coincident with apparently insignificant pH variations. Controlling the pH by means of M/500 buffer solutions stabilizes this variability.

2. Subjecting organisms of the colon-typhoid group to mild lethal conditions under moderate temperatures and hydrogen ion concentrations tends to magnify the induction period prior

<sup>6</sup> It must be remembered that the logarithmic curve merely integrates the results of all factors. It is a statistical summation and gives no information regarding the forces at play.

to mortality at the maximum or logarithmic rate. This provides an opportunity for studying the early response of the organism to the disinfection process.

3. The period of induction is decreased by higher acidity and by higher temperature. It appears to have a duration inversely proportional to some exponent of the temperature. It is analogous to the induction period occurring in chemical reactions.

4. At constant pH, the relative resistance of *Bact. coli* to *Bact. typhosum* decreases with rise in temperature from  $0^{\circ} : 10^{\circ} : 20^{\circ} : 30^{\circ}$  in the ratio of 67 : 51 : 18 : 8.

5. At  $20^{\circ}\text{C}$ . *Bact. typhosum* possesses the greatest tolerance within a narrow zone of hydrogen ion concentration delimited by pH 5.0 and 6.4. A slight increase in acidity beyond the zone results in conditions of maximum mortality. For *Bact. coli* the zone is wider and centered about absolute neutrality. Cohen and Clark (1919) found that the pH optima for growth and fermentation of bacteria may be different. It is now shown that the optimum for tolerance may also be distinct.

6. The mortality of bacteria whether by strong disinfectants or by milder agents follows the laws of logarithmic decline. It is shown that the course of the disinfection process can be expressed by mathematical relations comparable to those used in dealing with monomolecular chemical reactions.

I desire to express my cordial appreciation for important criticism and suggestions to Drs. C.-E. A. Winslow, E. Elvove, S. C. Brooks and especially to Dr. W. Mansfield Clark.

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## APPENDIX

In the following pages will be found, in tabular form, the detailed experimental results. Most of them have been presented in graphic form in the charts.

The values tabulated are the Briggsian logarithms of the numbers of survivors per cubic centimeter of water or buffer solution. Observations of the pH of the suspending fluid are noted wherever made.



*Experiment 1. Mortality of Bact. typhosum in double-distilled water in paraffin-lined bottles at different temperatures*

TIME	0°C.	10°C.		20°C.		30°C.	
	I	I	II	I	II	I	II
<i>hours</i>							
0	6 0133	6 2122	6 1399	6 0294	6 0569	6 1335	6 0969
pH	6.4	6.3	6.2	6.4	6.2	6.3	6.3
6	6 0038	6 1584	5 9085	6 0128	5 9754	6 0645	6 0645
pH	6.3	6.1	6.2	6.1	6.1	6.3	6.3
12	6 0112	6 1959	5 9004	6 0453	5 9814	5 9978	6 0086
24	6 0008	6 1038	5 8000		5 7882	6 0128	5 9685
72	5 9981	5 8209	5 5366	6 0064	5 7059	5 1847	5 6609
120	5 9780	5 5933	5 2304	5 7251	5 2672	3 0492	5 0934
pH	6.5	6.5	6.4	6.6	6.5	6.4	7.0
240	5 9664	4 8382	3 2014	4 8919	2 7076	0 7782	3 6201
pH	6.6	6.7	6.5	6.7	6.6	6.9	7.4
336	5 8782	3 4900	1 3802	3 3962	1 2553	Sterile	1 7634
	6.7	6.9	6.5+	6.8	6.6	7.0	7.5

*Experiment 2. Mortality of Bact. coli in double-distilled water contained in paraffin-lined bottles at different temperatures*

TIME	6°C.		10°C.		20°C.		30°C.	
	I	II	I	II	I	II	I	II
<i>hours</i>								
0	6 1430	6 4116	6 3351	5 7340	5 9619	5 8325	6 1103	5 9474
pH	6.1	6.5	6.4	6.3	6.3	6.1	6.3	6.2
24	6 0000	6 3617	6 1761	5 4425	5 7782	5 6096	5 8439	5 7076
48	5 9074	6 3234	6 1038	5 3617	5 7938	5 5366	5 8506	5 7235
72	5 8470	6 3962	6 0679	5 3522	5 7160	5 4871	5 7059	5 6794
120	5 8075	6 3040	6 1504	5 4472	5 6314	5 4848	5 4298	5 6551
240	5 5575	6 1895	5 9143	5 2227	5 3560	5 0170	5 1818	5 5877
pH	5.9	6.1						
360	5 1106	6 1096						
480	4 9410	6 0852	5 3181	4 8109	5 2330	4 8882	4 8055	4 6911
pH	5.8	6.3	6.4	6.2	6.4	6.3	6.4	6.3
600	4 3766	6 0311						
pH	6.0	6.5						
720			5 3075	4 3424	5 1367	4 7177	4 4082	4 1004
pH		6.8	6.3	6.3	6.5	6.5	6.6	6.5
1080			4 9455	3 9768	4 7435	4 5988	3 0899	3 1399
pH			6.3	6.3	6.4	6.3	6.1	6.2
1464			4 7931	3 8261	4 0607	4 4843	2 3909	2 7243
pH			6.5	6.5	6.7	6.7	6.6	6.5
1680			4 7435	3 5786	3 6263	4 3345	2 0000	2 5611
pH			6.3	6.5	6.7	6.6	6.8	6.7

*Experiment 3. Mortality of Bact. typhosum in autoclaved tap water at different temperatures*

TIME	0°C.	10°C.		20°C.		30°C.	
	I	I	II	I	II	I	II
<i>hours</i>							
0	5.9168	6.1875	6.2279	6.1492	5.8779	6.2648	6.2330
pH	9.4	9.3	9.4	9.4		9.3	
6		6.1399	6.1533	6.1206	5.8603	6.1732	6.2279
pH		9.1	9.2	9.2	9.2	9.3	9.3
12	5.7374	6.1303	6.1523	6.0494	5.8470	5.8149	5.8938
24	5.8000	6.0170	6.0294	5.9009	5.6821	4.7803	5.4871
72	5.4813	5.2504	4.6444	5.2175	4.9836	2.6551	2.6875
120	5.4009	5.1818	4.1523	4.8414	4.4728	2.1271	1.9542
pH	8.8	8.8	9.1	8.9	9.1	8.9	9.1
240	5.3372	4.3784	2.0607	3.9671	2.9128	1.2014	0.0000
pH	8.7	8.7	9.3	8.9	9.1	8.9	9.1
336	5.3195	4.0569	0.9031	3.6385	1.9031		
pH	8.7	8.8	9.3	8.9	9.2		

*Experiment 4. The mortality of Bact. coli at different temperatures in Berkefeld-filtered tap water*

TIME	0°C.		10°C.		20°C.		30°C.	
	I	II	I	II	I	II	I	II
hours								
0	6 2156	6 4267	6 0682	6 1315	6 0187	6 0278	5 8319	6 0682
pH	7.5	7.5	7.8	7.8	7.6	7.6	7.5	7.8
24	6 1697	6 4123	6 0800	6 0738	5 9557	5 9863	5 7513	6 0203
48	6 1967	6 3399	5 9782	6 0286	5 9191	5 9474	5 7917	6 0294
72	6 4212	6 3238	6 0064	6 0838	5 9727	5 9978	5 8082	5 9863
120	6 0856	6 3743	6 0382	6 0569	5 9600	5 8904	5 7745	5 9948
240	6 1196	6 3577		5 8028			5 7185	5 9868
pH	7.3	7.1						
360	6 0294			5 5611	5 9360	5 8537	5 4014	5 8692
pH				7.4	7.1	7.4	7.5	7.5
480	6 0766	6 4752		5 3096	5 7612	5 7664	4 7723	5 7543
pH	7.0	6.6		7.5	7.6	7.7	7.8	
600			Contami- nated	5 1732	5 5752	5 6335	4 0086	5 5922
pH				7.5	7.6	7.6	7.7	7.6
696	5 9445							
pH	6.7	6.6						
744				5 0531			2 7528	5 5159
pH					7.6		7.3	7.6
840	5 9777							
pH	7.0							
960				4 7033	4 6609	5 0569	0 0000	5 0864
pH				7.3	7.4	7.4	6.5	7.4
1200				4 5490	4 0294	4 1399	0 0000	4 9106
pH				7.5	7.6	7.5	7.0	7.5
1440				4 5092				
pH				7.7		7.7		

*Note:* The tap water was not subjected to high temperature to avoid dissolving glass constituents from containers.

*Experiment 5. The effect of hydrogen ion concentration upon the mortality of Bact. typhosum for forty-eight hours in M/500 buffer solutions at 20°C.*

TIME	pH 3.8		pH 5.0		pH 5.4		pH 6.4	
	I	II	I	II	I	II	I	II
hours								
0	5.3636	5.5682	5.7318	5.7825	5.7889	5.9036	5.8579	6.0000
3	2.3010	2.3010	5.6464	5.6212	5.7024	5.7686		
6	Sterile	Sterile	5.5623	5.5922	5.6875	5.6776	5.7118	5.7924
12			5.4425	5.4409	5.7059	5.7076	5.6637	5.7543
24			5.4031	5.4166	5.5038	5.7267	5.4518	5.5775
48			5.2041	5.0294	5.2672	5.3711	5.2253	5.3118
VELOCITY COEFFICIENT, <i>k</i>								
	1.0209	1.0891	0.0110	0.0157	0.0109	0.0111	0.0132	0.0143

TIME	pH 7.1		pH 7.6		pH 8.7		pH 9.5	
	I	II	I	II	I	II	I	II
hours								
0	5.8195	5.6730	5.8482	5.8414	5.5922	5.7093	5.5752	5.5775
3					5.0088	5.1818	5.1139	4.8082
6	5.8899	5.6180	4.9699	4.6284	4.4829	4.2148	4.0128	3.8096
12	5.8555	5.5478	4.6628	4.5441	3.3010	2.4771	2.0000	2.3010
24	5.5809	4.3997	2.9420	2.6675	1.0792	0.0000	0.0000	0.3010
48	4.6435	2.6532	0.0000	1.1139	Sterile	Sterile	Sterile	Sterile
VELOCITY COEFFICIENT, <i>k</i>								
	0.0244	0.0630	0.1215	0.0984	0.1887	0.2380	0.2979	0.2730

*Experiment 6. Mortality of Bact. typhosum in M/500 buffer of pH 3.5 at different temperatures*

TIME	0°C.		10°C.		20°C.		30°C.	
	I	II	I	II	I	II	I	II
minutes								
0	5.7760	5.8651	6.1004	6.0170	6.2856	5.9294	6.2041	5.9926
15					5.9106	5.4314	2.3617	2.0414
30			4.4533	4.1335	4.2695	4.6911	2.2625	1.6628
45					3.9854	3.6794	1.2788	1.0000
60	3.5065	3.8882	2.8865	3.3962	3.2455	3.1139	1.1461	0.6990
75						3.0000		
90			2.5911	2.9420				
120	1.8441	1.9294	1.9868	2.3032				
180	1.2788	1.0414	0.3010	0.3010				
240	1.1761	0.9777						
300	Sterile	0.0000						
VELOCITY COEFFICIENT, <i>k</i>								
	1.1500	1.2219	1.9931	1.9053	3.0401	2.8155	5.0580	5.2936

*Note:* The buffer consisted of 50 cc. M/5 potassium acid phthalate plus 14.7 cc. M/5 HCl in 5000 cc. distilled water. This had a pH of 3.2 which shifted to 3.5 during sterilization.

*Experiment 7. Mortality of Bact. coli in M/500 buffer of pH 3.5 at different temperatures*

TIME	0°C.		10°C.		20°C.		30°C.	
	I	II	I	II	I	II	I	II
<i>hours</i>								
0	6 1817	6 2648	6 2301	6 2155	6 0344	6 2455	6 2227	6.1703
1							5 9309	6.0253
2					6 0212	6 0607	5.0128	5 6415
3							4 9699	5 2718
4	6 1553	6 2227	6 1703	6 0492	5 6675	5 9063	4 3636	4 7627
5							3.4456	3.9590
6							2 7186	2 9289
8	6 0828	6 2227	6.0899	5 9590	5 2175	5 5092	0 6021	
12	6 0334	6.1599	5 9699	5.7007	4.0864	4 2253		
15	6.0212	6 1761	5 9117	5 3139				
24	5.7846	5 9538	5.5092	4 9186				
32	5 5478	5 7796	5 2856	4.8055				
VELOCITY COEFFICIENT, <i>k</i>								
	0 0199	0 0152	0 0295	0.0150	0.1623	0.1684	0.7026	0 5402

Note: The buffer solution was identical with that used in experiment 6.

*Experiment 8. The mortality of Bact. coli at different temperatures in M/500 buffer at pH 6.1*

TIME	0°C.		10°C.		20°C.		30°C.	
	I	II	I	II	I	II	I	II
<i>hours</i>								
0	6 2511	6 4578	6.0864	6 5824	6.4133	6 4249	6 1775	6 1790
24	6 1134	6 4305						
50	6 1271	6 4301						
74	6 0899	6 4085						
120	6 0846	6 4698	5 9180	6 5617	6 3858	6 3786	6 0676	6 1265
240	6 0933	6 4288	5 9325	6 5023	6 4171	6 3655	6 0660	6 0702
360	5 9357	6 2703	5 8848	6 4183	6 3416	6 2878	6 022	6 0212
480	5 9722	6 3049	5 7582	6 4232	6 3232	6 2844	5 9058	5 7042
600			5 8082	6 4198	6 2122	6 2148	5 5809	5 2625
720	5 8839	6 3219	5 7627	6 3670	6 2227	6 2068	5 3444	4 9047
810	5 9672	6 3959	5 6839	6 3579	6 0029	6 0128	5 2480	4 7679
960			5 7143	6 3619	6 1810	6 1422	4 9934	4 5587
1128			5 7152	6 4188	6 0745	6 0993	4 7185	4 3324
1230			5 6821	6 3275		6 0374	4 4440	4 1614
1440					5 9652			
1560			5 5416	6 1461	5 8998	5 8357	4 1072	3 8831
VELOCITY COEFFICIENT, <i>k</i> · 10 <sup>5</sup>								
			0 3154	0 2800	0 3292	0 3775	1 326	1 895

Note: The buffer consisted of 50 cc. M/5 KH<sub>2</sub>PO<sub>4</sub> plus 4.75 cc. M/5 NaOH in 5000 cc. distilled water. The original pH of 5.9 shifted to 6.1 during sterilization.

Experiment 9. The mortality of *Bact. coli* at different temperatures in M/500 buffer at pH 7.1

TIME	0°C.		10°C.		20°C.		30°C.	
	I	II	I	II	I	II	I	II
<i>hours</i>								
0	6.3701	6.4752	6.3811	6.4514	6.5316	6.2405	6.3113	6.5051
25			6.3304	6.4440	6.4984	6.2896	6.2516	6.4786
48			6.3560	6.3574	6.4422	6.2350	6.2417	6.4735
74	6.3369	6.4734	6.2863	6.4019	6.4393	6.2054	6.2169	6.4824
120	6.2941	6.4914	6.3218	6.4084	6.4764	6.2222	6.2300	6.4433
240	6.3707	6.4436	6.2941	6.3516	6.4777	6.1945	6.1897	
384	6.3287	6.4541	6.3240	6.3104	6.3647	6.0682	6.0708	6.2601
480	6.3072	6.4254	6.2397	6.3118	6.3879	6.0792	5.8248	5.9643
600	6.3131	6.4079	6.3047	6.3843	6.2471	5.9294	5.4843	5.6712
840	6.2922	6.3728	6.1553	6.2989	6.2034	5.9015	5.0864	5.2363
984			6.1239	6.3560		5.8451	4.9395	5.2455
1200			6.1453		6.0998	5.5366	4.7723	5.0374
1440			6.1106	6.2227	5.9041	5.3874	4.5855	4.8785
VELOCITY COEFFICIENT, $k \cdot 10^3$								
	0.0928	0.1220	0.188	0.159	0.436	0.593	1.20	1.13

Note: The buffer consisted of 50 cc. M/5  $\text{KH}_2\text{PO}_4$  plus 29.63 cc. M/5 NaOH in 5000 cc. distilled water. The original pH of 7.0 shifted to 7.1 during sterilization.

Experiment 10. The mortality of *Bact. coli* at different temperatures in M/500 buffer at pH 8.0

TIME	0°C.		10°C.		20°C.		30°C.	
	I	II	I	II	I	II	I	II
<i>hours</i>								
0	6.1818	6.4526	6.3892	6.3485	6.5498	6.4439	6.4417	6.4882
24	6.1120	6.3141	6.2856	6.3464	6.3575	6.2923	6.2487	6.2777
48	6.1139	6.3395	6.2853	6.2889	6.3187	6.2350	6.1281	6.1351
75	6.0503	6.2413	6.2826	6.2084	6.2041	6.1383	5.9759	6.0374
120	6.0128	6.2227	6.2608	6.2209	6.1351		5.9309	6.0000
240	6.0212	6.2034	6.0958	6.1106	6.0212	5.9624	5.7016	6.0086
360	5.9542	6.2470	6.1367	6.1523	6.0425	5.7825	5.5250	5.8351
480	6.0029	6.2427	6.1470	6.1162	6.0107	5.7657	5.2504	5.6464
600			5.9881	6.0294	5.9661	5.6911	5.1875	5.4518
720	6.0238	6.2365	5.9445	6.0199	5.8865	5.5786	4.8825	5.3243
840			5.8651	5.9633	5.8451	5.5119	4.6503	5.2601
960			5.9206	6.0098	5.9143	5.6981	4.5453	5.2577
1200			5.9243	6.0029	5.8082	5.5933	4.0229	5.1430
1440			5.8938	5.9912	5.7745	5.3032	3.4787	3.7067
VELOCITY COEFFICIENT, $k \cdot 10^3$								
	0.2194	0.3141	0.3441	0.2841	0.5385	0.7925	2.058	1.933

Note: The buffer consisted of 50 cc. of M/5  $\text{KH}_2\text{PO}_4$  plus 46.80 cc. M/5 NaOH in 5000 cc. distilled water. After sterilization, the pH remained at 8.0. Toward the end of the experiment, the pH gradually shifted to 7.7, apparently because of the low concentration of the buffer and the nearness to the limit of its zone of effectiveness.

Experiment 11. Mortality of *Bact. coli* in *M/500* buffers at 20°C.

TIME	pH 8.4		pH 8.9		pH 9.5	
	I	II	I	II	I	II
hours						
0	6.5391	6.2584	6.3874	6.4564	6.1818	6.2577
24	6.5159	6.2430	6.3973	6.3079	6.1703	6.1206
48	6.4683	6.2114	6.3784	6.4502	6.0719	6.0294
72	6.6021	6.2492	6.4031		6.0374	6.0607
120	6.4969	6.1614	6.4216	6.4654	6.0607	5.9638
264	6.4393	6.2072	6.4089	6.4314	5.9890	6.0086
480	6.5809	6.2227	6.5172		5.9890	6.0098
624	6.5539	6.2227	6.5441	6.4624	5.9912	5.7973
840	6.4955	6.2201	6.4133	6.3711	5.8494	5.8573
	pH shifted to 7.9					

Experiment 12. The mortality of *Bact. typhosum* in autoclaved tap water at 20°C.

TIME		I	II	III
hours				
0		6.5873	6.5628	6.7284
	pH	8.9	8.7	8.4
6		6.5463	6.5721	6.6887
	pH	8.9	8.7	8.4
24		6.4742	6.5575	6.6939
	pH	9.0	8.8	8.8
48		5.1931	6.5968	6.6532
	pH	8.9	8.8	8.7
72		3.4914	5.2742	6.6135
	pH	8.9	8.7	8.7
96		2.6096	4.9074	6.5064
	pH	8.9	8.7	8.6
120		2.4065	4.6375	6.4389
	pH	8.9	8.7	8.5
144		2.2201	4.7771	6.2695
	pH	8.9	8.7	8.6
167		1.9243	4.2380	6.1804
	pH	8.9	8.5	8.5
216		1.1139	3.6021	5.6821
	pH	8.5	8.3	8.2
264		0.6990	5.7275	5.5159
	pH	8.2	7.7	7.6
312		Sterile	5.6096	5.2684
	pH	8.1	7.9	7.9
384			5.4654	4.9299
		8.3	8.1	8.1

*Experiment 13. Mortality of Bact. typhosum in M/500 buffer of pH 6.3-6.5 at 20°C.*

TIME	PHOSPHATE BUFFER			PHTHALATE BUFFER		
	I	II	III	I	II	III
<i>hours</i>						
0	6.6133	6.5376	6.5417	6.6073	6.5234	6.4469
6	6.6314	6.5703	6.5931	6.6703	6.5289	6.4510
24	6.6583	6.5090	6.5916	6.6086	6.5489	6.4713
48	6.5991	6.5714	6.5895	6.6074	6.4821	6.4004
72	6.5705	6.5907	6.5844	6.6425	6.4938	6.4327
96	6.6087	6.5609	6.5585	6.5821	6.5314	6.4284
120	6.5627	6.5038	6.4813	6.4846	6.4705	6.4480
144	6.5714	6.5502	6.5370	6.5555	6.4548	6.3720
167	6.5991	6.5658	6.5658	6.4705	6.4702	6.3988
216	6.5363	6.5502	6.3375	6.4975	6.3756	6.3290
264	6.5647	6.5563	6.5922	6.5350	6.4487	6.4294
312	6.6206	6.5723	6.6157	6.5488	6.4932	6.3639
384	6.5899	6.5447	6.5465	6.5798	6.5452	6.4396
552	6.2876	6.4976	6.6204	6.4343	6.3897	6.2819
763	5.5872	5.9064	5.9694	5.9890	6.0174	5.6945
960	5.0515	5.3345	5.7179	5.7669	5.8873	5.2978

*Note:* The Clark and Lubs phosphate and phthalate buffers of pH 6.0 were diluted to M/500 and autoclaved, after which their pH remained between 6.3 and 6.5. This experiment was performed six months after Nos. 1-11 and shows that any specific effect of the buffer as a salt is subordinate to the pH effect, at least under these conditions.



# MICROÖRGANISMS CONCERNED IN THE OXIDATION OF SULFUR IN THE SOIL

## I. INTRODUCTORY<sup>1</sup>

SELMAN A. WAKSMAN

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Hydrogen sulfide and other sulfides in solution are slowly oxidized to sulfur, under natural conditions. The sulfur formed, as well as elementary sulfur, particularly when present in a fine state of subdivision and in the presence of certain catalytic agents, undergoes further oxidation, the resulting product being sulfuric acid. In the presence of specific bacteria, this phenomenon is much more rapid. These bacteria were first studied extensively by Winogradsky and were designated by him as sulfur bacteria; they possess a strong oxidizing power, in contradistinction to the reducing bacteria, which form hydrogen-sulfide from sulfur and its compounds. The sulfur bacteria, originally studied by Winogradsky, contain sulfur granules within their cells, as a result of the oxidation of the hydrogen sulfide. Later investigators, in their studies of sulfur oxidation, also included, under the term sulfur bacteria, organisms which are able to oxidize hydrogen sulfide, sulfur, and thiosulfate, but which do not, however, store any sulfur within their cells.

The so-called sulfur bacteria are not related to one another morphologically and belong to widely different genera. By the use of physiological and morphological differences, they can be divided into five groups: (1) colorless, thread forming bacteria, accumulating sulfur within their cells. (2) Colorless, non-thread-forming bacteria, accumulating sulfur within their cells. (3) Purple bacteria, oxidizing sulfur and accumulating it within

<sup>1</sup>Paper No. 32 of the Technical Series, New Jersey Agricultural Experiment Station, Department of Soil Chemistry and Bacteriology.

their cells. (4) Bacteria oxidizing sulfur and sulfur compounds, but accumulating sulfur outside their cells. (5) Bacteria oxidizing elementary sulfur and not accumulating any sulfur within or without their cells. The first four groups are mentioned in the latest texts and reviews on sulfur bacteria, while the fifth group has been suggested on the basis of the results presented below.

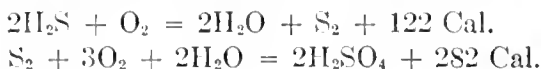
It is of interest to note that in most of the work on sulfur oxidation by bacteria, with the exception of that of Jacobsen (1912),<sup>2</sup> the starting point was not sulfur itself, but hydrogen sulfide, sulfides, or thiosulfates. The activity of the organisms was judged not by the oxidation of sulfur, as measured by the production of sulfuric acid and sulfates and subsequent change in reaction, but either by the disappearance of the sulfide in the medium or by the appearance or disappearance of the sulfur granules within or without the microbial cell. Of importance is also the fact that all the earlier work and most of the later work has been done with organisms present in canal water, mud water, curative muds, and sea water and very little attention was paid to the microorganisms concerned in the oxidation of sulfur in the soil. This is the reason for using hydrogen sulfide and sulfides as a source of sulfur, since the latter substance, and not elementary sulfur, is present or is produced abundantly under those conditions.

A detailed review of the oxidation of sulfur and sulfur compounds by microorganisms is given in the papers of Omeliansky (1904), Duggeli (1919) and in the book by Kruse (1910). A brief historical review is presented here for a better understanding of the work that is to follow.

The first group of sulfur oxidizing bacteria (colorless, thread forming) consists of three genera: *Beggiatoa*, motile, forming no sheaths; *Thiothrix*, fastened, forming no sheaths, and *Thioploca*, threadforming bacteria, surrounded with a jelly-like sheath. The *Beggiatoas* were the first organisms to attract attention as having to do with the oxidation of sulfur or its derivatives. Cramer (1870) pointed out that the granules found

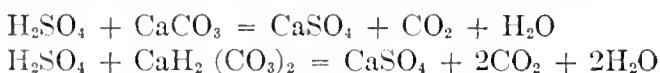
<sup>2</sup>Bibliography is found at the end of the second article, in this series.

within the cells of *Beggiatoa* consisted of sulfur. Cohn (1875) then proposed the theory that the *Beggiatoa* and the purple bacteria produce hydrogen sulfide by the reduction of sulfates. But it was Winogradsky (1883, 1887, 1888) who demonstrated that the hydrogen sulfide produced by other bacteria is oxidized by the *Beggiatoa* to sulfur and sulfuric acid.



This oxidation is so important for the very existence of these organisms that, when the hydrogen sulfide is taken out of the medium, they oxidize the sulfur present within their cells and, when this is used up, they die out. The presence of traces of organic substances and nitrates in the water is sufficient for the development of these organisms, as long as there is enough hydrogen sulfide, while the presence of sugars, peptone and like nutrients will stimulate the growth of other microbes but will injure these sulfur bacteria.

According to Winogradsky (1888), the sulfuric acid formed is neutralized by the calcium carbonate present in the water.



The reaction of the water cultures of the acid producing bacteria was not found to become acid. *Beggiatoa* has been obtained in pure culture by Keil (1912). No definite physiological studies were made by Winogradsky. In general, the results of this investigator are summarized under the following four headings: (1) The sulfur bacteria oxidize hydrogen sulfide and accumulate sulfur in the form of small spheres, consisting of soft amorphous sulfur which never crystallizes in the living cells. (2) They oxidize the sulfur to sulfuric acid, which is at once neutralized, by the carbonates present, into sulfates. (3) Without sulfur, the organisms soon die off. (4) They can live and multiply in liquids containing only traces of organic substances.

This last point was refuted by Keil (1912), who demonstrated that the organisms are autotrophic and do not need organic

substances for their growth. Keil claims to have isolated pure cultures of *Beggiatoa* and *Thiothrix*, and found these organisms to be able to live in media free from any traces of organic matter, although the presence of small quantities of organic substances is not detrimental to these organisms. Ammonium salts are used as sources of nitrogen and only carbonic acid as a source of carbon. Carbon dioxide pressure may vary within the limits of 0.5 and 350 mm. (25 mm. is the optimum); oxygen may vary within 10 to 20 mm. and  $H_2S$  within 0.6 to 1.7 mm. The presence of carbonates is important for the neutralization of the acids. The organism seems to assimilate carbon at the rate of 1 gram per 8 to 10 grams of sulfur oxidized (see also Hinze and Molisch). The *Thioplaca* has been studied in detail by Wisloch and Kolkwitz.

The second group of the sulfur oxidizing bacteria consists of colorless organisms forming no threads and containing sulfur within their cells. The following forms belong to this group: *Monas* (Hinze (1913)), *Thiophysa* (Hinze (1903)), *Thiovulum* (Hinze (1913)), *Spirillum* (Molisch (1912)), *Thiospirillum* (Omelianski (1905)), *Bacterium bovista* (Molisch (1912)), *Bacillus thiogenes* (Molisch (1912)), and *Achromatium* (Nadson (1903), and Griffith (1913)).

Some idea of this group of organisms is obtained from a reference to the work of a few investigators. Jegunow (1896) studied the oxidation of the hydrogen sulfide formed in the mud of the Liman in Odessa and in the Black Sea. He described two sulfur bacteria; *Thiobaeterium*  $\alpha$ , a motile, colorless, slightly curved organism, 4.5 to 9 $\mu$  long and 1.4 to 2.3 $\mu$  wide, containing a finely granulated plasma and large sulfur granules. *Thiobaeterium*  $\beta$ , motile, colorless, curved, 2.5 to 5 by 0.6 to 0.8 $\mu$ , and containing a row of shining sulfur granules. *Monas Müllerii* was described in detail by Hinze (1913), who has shown that this organism belongs morphologically to the flagellates and physiologically to the sulfur bacteria. Most of these organisms were isolated from the water.

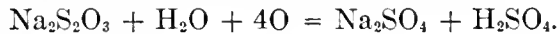
Gicklehorn (1920) recently described several new sulfur bacteria, of which two are classified with this group, namely: *Spiril-*

*lum agilissimum* isolated from river mud in Gratz which measures about 6 to 10 by 1.8 to 2 $\mu$ , of a rapid motility, and filled with black sulfur granules; *Chromatium cuculliferum* which is round to slightly elliptical, 6 by 4 $\mu$ , of a slow motility, with black, shining, sulfur drops always found in one pole, with one flagellum on the granule-free pole. This form was found in a rotting mass of algae in the garden basin at Gratz. Gicklehorn also described three more forms: *Miospirira vacillans*, *Pseudomonas bipunctata* and *Pseudomonas hyalina*, observed in the slime of the large basin in the botanical garden at Gratz, which he classified with the colorless sulfur bacteria. It must be noted here that none of the forms studied by Gicklehorn were cultivated in pure culture, which is true of most of those studied by the other investigators.

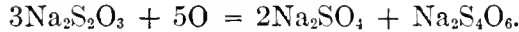
The third group of the sulfur oxidizing organisms is found among the purple bacteria. These are distinguished from the sulfur bacteria described above by the production of a red, red violet or red brown pigment which is unevenly distributed throughout the cell. In addition to the red pigment (bacterio-purpurin), there is also present in all these bacteria a green pigment (bacterio-chlorin). These bacteria are found abundantly in sulfur springs and in mud waters. Not all the purple bacteria are able to utilize hydrogen sulfide. Molisch (1907) succeeded in cultivating some of them in pure culture, but not the sulfur forms. The rôle of sulfur in the metabolism of purple bacteria is still an open question, since, according to Nadson (1903) and Molisch (1907), the hydrogen sulfide is not required for nutrition and sulfur is not accumulated. These results are in direct opposition to the earlier ideas of Winogradsky (1888) and others. A detailed study of the purple bacteria is found in the work of Molisch (1907).

The fourth group of sulfur bacteria includes colorless organisms that do not accumulate sulfur within their cells. These were first demonstrated by Nathanson (1903) in sea water, and were found to be able, by means of oxidation of hydrogen sulfide or sodium thiosulfate, to reduce carbonic acid and construct from it organic substances. By using a medium consisting of 3 per cent NaCl, 0.25 per cent MgCl<sub>2</sub>, 0.1 per cent KNO<sub>3</sub>, 0.05 per cent

$\text{Na}_2\text{HPO}_4$ , 0.2 to 1 per cent  $\text{Na}_2\text{S}_2\text{O}_3$  and some  $\text{MgCO}_3$ , he obtained a good growth of these bacteria and, on adding agar, he has been able to isolate them in pure culture. In the absence of the carbonate, but in the presence of  $\text{CO}_2$  containing air, the growth was much slower; in the absence of both carbonate and  $\text{CO}_2$ , no growth took place, even in the presence of various organic substances. The medium did not become acid even in the absence of carbonate, indicating that the oxidation of the thiosulfate did not take place according to the formula:

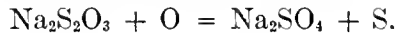


but according to the following reaction:



While no sulfur accumulates within the cell, there is an abundant production of free sulfur outside of the cell, not in direct contact with the colony, but at some distance from it, suggesting an extracellular oxidation.

Beijerinck (1904) confirmed the results of Nathanson (1903) by the use of a medium consisting of 100 parts of water, 0.5  $\text{Na}_2\text{S}_2\text{O}_3$ , 5  $\text{H}_2\text{O}$ , 0.1  $\text{NaHCO}_3$ , 0.02  $\text{K}_2\text{HPO}_4$ , 0.01  $\text{NH}_4\text{Cl}$  and 0.01 part  $\text{MgCl}_2$ . The medium was not sterilized, was inoculated with canal water and incubated at 28 to 30°. In 2 to 3 days, the surface of the medium became covered with free sulfur, filled with bacteria. On making a transfer into a fresh flask with medium, a sulfur layer was obtained in 24 hours. According to Beijerinck (1904), the reaction takes place as follows:



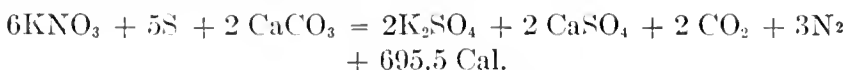
This reaction is exothermic and functions as a source of energy, which is used for the reduction of  $\text{NaHCO}_3$  and for the building of the bacterial body.  $\text{CaS}$  and  $\text{H}_2\text{S}$  can replace the thiosulfate.  $\text{H}_2\text{S} + \text{O} = \text{H}_2\text{O} + \text{S}$ .



The ammonium salt can be replaced by nitrates. No other of the organic substances tested could replace the carbonic acid as

a source of carbon. The organism, *Thiobacillus thioparus*, is a short rod, 3 by 0.5 $\mu$ , not forming any spores, very motile and very sensitive, so that on plates the organisms die off in a week.

By using a medium consisting of canal water 100, powdered sulfur 20, KNO<sub>3</sub> - 0.5, Na<sub>2</sub>CO<sub>3</sub> - 0.02, CaCO<sub>3</sub> - 2.0, K<sub>2</sub>HPO<sub>4</sub> - 0.02 parts, in a closed flask, incubated at 30°C., Beijerinck obtained an oxidation of sulfur accompanied by a reduction of the nitrate to atmospheric nitrogen.



The sulfur is oxidized to sulfuric acid which acts upon the CaCO<sub>3</sub> giving CaSO<sub>4</sub> and CO<sub>2</sub>. By using a medium consisting of tap water - 100, Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> · 5 H<sub>2</sub>O - 0.5, K<sub>2</sub>HPO<sub>4</sub> - 0.01, NaHCO<sub>3</sub> - 0.02, agar 2.0 parts, Beijerinck isolated, in pure culture, the organism, *Thiobacillus denitrificans*, which is a very motile, short rod, hardly distinguishable microscopically from *Thiobacillus thioparus*. *Thiobacillus denitrificans* was further studied by Lieske (1912) and Gehring (1915) and was found to occur in various soils. The organisms on the plate, lose their ability to grow rapidly, long before they are dead. Beijerinck's work was continued further by Jacobsen (1912, 1914), who found a crude culture of *Thiobacillus thioparus* to be able to oxidize 58.8 mgm. of sulfur to sulfuric acid, in five weeks, out of a total of 648 mgm. added to the medium. Pure cultures oxidized, in eight weeks, 165 mgm. out of 648 mgm. of sulfur added.

Gicklehorn (1920) has studied two organisms belonging to the fourth group of sulfur-oxidizing bacteria, found in garden soil, which are able to oxidize K<sub>2</sub>S with the liberation of free sulfur. The organisms are 1 to 2 by 0.3 to 0.5 $\mu$  and 2 to 4 by 0.5 to 1 $\mu$  in size. However, he did not isolate his organisms in pure culture and did not record any quantitative physiological data.

Finally, we have a fifth group of sulfur bacteria, which are studied in detail in the next paper. Two preliminary reports on this organism by Waksman and Joffe (1921a, 1921b) and a detailed study of the methods used in its isolation, by Lipman, Waksman and Joffe (1921), were published elsewhere.

To summarize: (1) The microorganisms concerned in the sulfur cycle are separated into reducing bacteria and oxidizing bacteria, the latter being the true sulfur bacteria. (2) The true sulfur bacteria are divided into five groups: the first three groups of sulfur bacteria are found in sulfur springs, canal and mud waters, curative muds, river water and sea water; they oxidize hydrogen sulfide and sulfides, but not elementary sulfur, and accumulate sulfur within their cells; the fourth group of bacteria, consisting of small rod shaped organisms, is found in sea water, canal water and soil; these bacteria are able to oxidize hydrogen sulfide and other sulfides, thiosulfates and elementary sulfur, forming a heavy pellicle on the surface of the medium and allowing an accumulation of sulfur outside of their cells; the fifth group of sulfur bacteria occurs in soils to which elementary sulfur has been added, particularly in soil-sulfur-composts, oxidizing primarily elementary sulfur, thiosulfates to a small extent, but not hydrogen sulfide or sulfides; these bacteria grow uniformly throughout the medium, not forming any pellicle, do not liberate any sulfur and allow a very intensive production of sulfuric acid, and the necessary carbon is derived entirely from the carbon dioxide of the atmosphere; the fifth group is morphologically related to group four, but includes organisms very small in size and the strongest sulfur oxidizing and acid producing bacteria known.



# MICROÖRGANISMS CONCERNED IN THE OXIDATION OF SULFUR IN THE SOIL

## II. THIOBACILLUS THIOOXIDANS, A NEW SULFUR-OXIDIZING ORGANISM ISOLATED FROM THE SOIL<sup>1</sup>

SELMAN A. WAKSMAN AND J. S. JOFFE

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By composting sulfur, rock phosphate and soil it was found (McLean, 1918) that sulfur is rapidly oxidized to sulfuric acid; the acid acts upon the tricalcium phosphate, converting it into di- and mono-calcium salts. In the absence of a neutralizing agent or, after this agent has all been used up, the sulfuric acid formed, in the presence of an excess of sulfur, accumulates in the medium. On inoculating such composts into proper culture media, we finally succeeded in isolating a small bacterium which is active in the oxidation of the sulfur. A detailed study of the composting of sulfur, of the transformation of the tri-calcium phosphate and of the methods used in the isolation of the organism are found elsewhere (Lipman, Waksman and Joffe, 1921); only a brief review of the process of isolation is presented here.

*Method of isolation.* The following media were originally used for the isolation of the organism:

*Medium 1:*

(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> .....	2.00 gram
K <sub>2</sub> HPO <sub>4</sub> .....	1.00 gram
MgSO <sub>4</sub> .....	0.50 gram
KCl.....	0.50 gram
FeSO <sub>4</sub> .....	0.01 gram
Sulfur.....	10.00 grams
Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub> .....	10.00 grams
Distilled water.....	1000.00 cc.

*Medium 2:* Same as no. 1, but with 0.1 per cent glucose.

*Medium 3:* Same as no. 1, but in place of 10 grams only 2.5 grams Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> ✓  
per liter.

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<sup>1</sup>Paper No. 33 of the Technical series, New Jersey Agricultural Experiment Stations, Department of Soil Chemistry and Bacteriology.

The media were distributed in 100 cc. portions into 250 cc. Erlenmeyer flasks and sterilized in flowing steam, for 30 minutes, on three consecutive days. The flasks were then inoculated with various dilutions of the composts. Medium 2 was found to allow a growth of both a sulfur oxidizing bacterium and one or more species of fungi. By omitting the glucose from the medium, the fungi were practically eliminated.

It was found later that, by cutting down the tri-calcium phosphate in the medium to 0.25 per cent, a more rapid development of the organism took place, thus giving medium 3, which is a modification of 1 and 2.

Well advanced composts were used for inoculation. The material was diluted 10, 1000, 100,000 and 10,000,000 times with sterile water, then 1 cc. of each dilution was added to 100 cc. of the sterile medium and the flasks incubated, at 25°, for seven to fourteen days.

The flasks became turbid on the fourth or fifth day, the amount of turbidity depending upon the dilutions used, the higher dilutions developing slower than the lower ones. A pellicle or fungus mycelium was formed only in the flasks containing glucose. By transferring the cultures into fresh flasks, the same phenomenon was observed with a uniform turbidity in four to five days. By examining the culture under the microscope, it was found to contain a very minute non-motile bacterium present in abundance and accompanied by a few larger cylindrical cells which were found to be spores of a fungus occurring abundantly in the compost. The impure culture of the organisms was found to possess strong sulfur-oxidizing properties, about 200 to 300 mgm. of the sulfur being oxidized, in each flask, in fourteen days. In the presence of tri-calcium phosphate more of the sulfur is oxidized, since the acid formed is used up in converting the insoluble phosphate into soluble calcium-acid-phosphate and calcium sulfate. A further accumulation of the sulfuric acid resulted also in the formation of phosphoric acid and calcium sulfate. The medium had originally a reaction equivalent to pH 5.6 to 6.2. Following the oxidation of the sulfur, the reaction became gradually acid and, at a pH of

2.6-2.8, the reaction remained stationary till all the tri-calcium phosphate had been transformed into mono-calcium salt, after which the reaction became more acid, as shown in table 1 and figure 1.

All attempts to grow the sulfur-oxidizing organisms on solid media failed, neither agar nor silica-jelly media allowing any growth to take place.

TABLE 1  
*Course of reaction and accumulation of water soluble phosphates*

AGE OF CULTURE	pH	PER CENT OF INSOLUBLE PHOSPHATES MADE WATER SOLUBLE*
<i>days</i>		
At start	5.4	
1	5.4	
2	5.3	0.9
4	4.6	5.5
6	3.5	
8	2.6	33.7
10	2.7	27.5
12	2.6	81.7
15	2.4	93.9
19	2.3	86.3
23	2.3	85.9
30	1.8	
38	1.8	86.1
68	1.7	
120	0.8	85.9

\*Medium contains originally 1 per cent insoluble phosphate.

A pure culture was obtained by continued transfer in fresh flasks with high dilutions, so as to eliminate any contaminating organism, the medium being made acid at the start (pH 2.0-3.0), by the use of phosphoric acid and mono-potassium phosphate. The culture was finally obtained in a pure state. Its purity was demonstrated by repeated microscopic examinations, by the uniform growth in the liquid media and by the fact that no organism developed, when the culture was inoculated upon common bacteriological media.

On repeated transfer, the culture was found to deteriorate since it took a longer period of time to develop. It was found, necessary, in order to obtain a good growth, to use a sterile pipette instead of a loop, one or two drops being sufficient to inoculate 100 cc. By buffering the medium with suitable substances, such as phosphates, the organism would develop much more rapidly, particularly at the more acid reactions. The

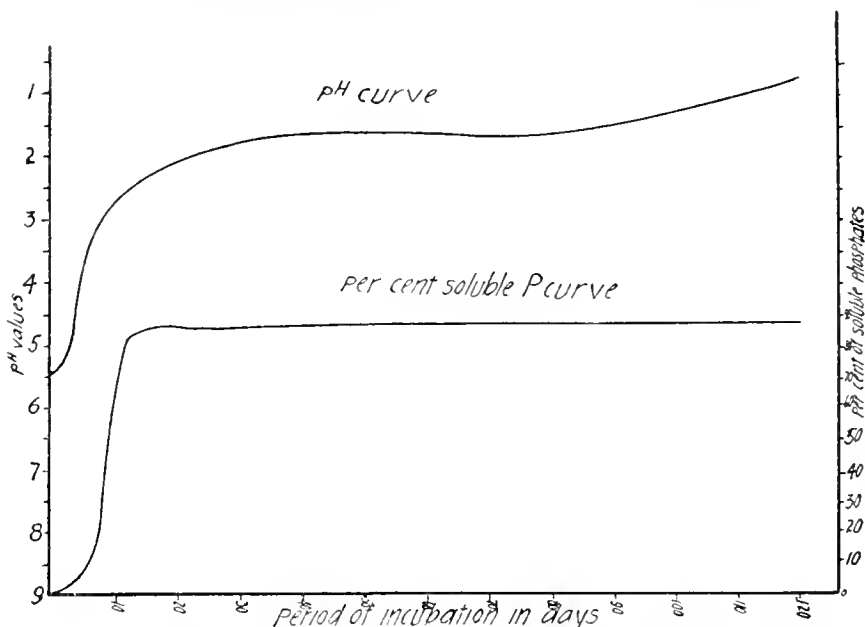


FIG. 1. COURSE OF REACTION AND ACCUMULATION OF WATER SOLUBLE PHOSPHATES IN A PURE CULTURE OF *Thiobacillus thiooxidans* N. SP.

organism was found to be morphologically similar to the two *Thiobacilli* described by Beijerinck, and it is, therefore, classified in that genus, under the name of *Thiobacillus thiooxidans* n. sp.

*Morphology.* Vegetative cells, on the synthetic media used, are short rods, with rounded ends, usually occurring singly, to some extent in pairs and rarely in triplets. The majority are less than 1 micron long and about 0.5 micron in diameter. Spore formation, absent. The majority of the cells are non-motile, although a few motile cells can also be found in young (seven

days old) cultures. The organism stains well with gentian-violet and methylene blue. It is Gram-positive.

#### CULTURAL CHARACTERISTICS

No agar or other solid medium has been found as yet, upon which the organism would grow. It grows in liquid media with a strong uniform clouding, without any surface growth or sediment formation. It does not grow on the common organic media, although the presence of glucose or peptone in the medium is not injurious. Inorganic media containing sulfur as a source of energy are suited for its growth. In the presence of tri-calcium phosphate, the growth of the organism is accompanied by characteristic reactions: the sulfur forming originally a layer on the surface of the medium usually drops to the bottom, the sulfuric acid formed from the oxidation of the sulfur dissolves the tri-calcium phosphate giving soluble phosphate and  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ , the calcium sulfate crystallizes out in the form of radiating monoclinic crystals hanging down from the sulfur particles that are floating on the surface of the medium or protruding upward from the bottom. The reaction of the medium becomes acid as indicated by the change in the hydrogen-ion concentration. At a pH of about 2.8, the reaction becomes stationary till all the calcium-phosphate has been dissolved. In the presence of an excess of this neutralizing agent, or in the presence of rapidly dissolving alkaline carbonates, the culture is injuriously affected. Anything that will tend to change the medium to an alkaline or even a less acid reaction (except, of course, the action of the buffers), such as shaking the culture, in the presence of even smaller amounts of tri-calcium phosphate, will also tend to affect the uniform growth of the organism injuriously.

The culture can be kept alive for numerous consecutive generations on the liquid media and when not injured by an excess of alkali or acid, may be as active as a recently isolated culture.

The index No. of *Thiobacillus thiooxidans* is, according to the new Descriptive Chart of the Society of American Bacteriologists, 5332-5230-2222.

## PHYSIOLOGY

*Source of carbon.* The organism derives all its carbon need from the  $\text{CO}_2$  of the atmosphere. When carbon was introduced into the culture in the form of carbonates and bicarbonates, the presence of the former prevented growth due to the fact that they kept the medium alkaline, thus preventing a normal development of the organism, while the latter, if present only in small amounts, allowed a good growth to take place. But since the growth was not any better, and to some extent even worse than in the bicarbonate-free flasks, its use is superfluous. At this point, we get a clear differentiation in the metabolism of two important autotrophic organisms, the nitrifying and the sulfur-oxidizing bacteria. While the former thrives best at an alkaline reaction, the latter grow best at an acid reaction. Sodium bicarbonate is considered to be indispensable for the nitrifying bacteria; this was thought to be due to the utilization of the bicarbonate as a source of carbon, but, as recently pointed out by Meyerhof (1916), the bicarbonate merely serves the purpose of a buffer in the medium, to keep the reaction alkaline (optimum pH 8.3-9.3). In the case of the sulfur oxidizing bacterium, which has its optimum at a distinctly acid reaction (pH 3.0-4.0), the bicarbonate is not necessary since its buffering properties will tend to make the medium less acid and thus have an injurious effect, while as a source of carbon, the  $\text{CO}_2$  from the atmosphere seems to be sufficient.

*Source of energy.* Sulfur is the all important source of energy for this organism. The organism is strictly autotrophic and, although glucose did not exert any injurious action, and perhaps its action was even to some extent beneficial, the amount of sulfur oxidized and acid produced were about the same in glucose and in glucose-free cultures.

In addition to sulfur, thiosulfate is also utilized, but to a much smaller extent: while, with elementary sulfur, growth appears in four to five days, under favorable conditions, as demonstrated by the turbidity and change in pH value, with thiosulfate, growth appears only in ten to twelve days and is much slower.

Hydrogen sulfide and sulfides are not utilized at all, which sharply differentiates our organism from those of Nathanson (1903), Beijerinck (1904), and Jacobsen (1914), as will be pointed out later.

*Mineral requirements.* Mere traces of K, Mg, Ca, Fe, in addition to phosphates, are sufficient for the growth of the organism. As a matter of fact, good growth and good sulfur oxidation were obtained by omitting, in various batches of media, each of the first four minerals, but, of course, no precaution was taken to eliminate any traces present in the distilled water or any substances that might have been dissolved out by the action of the sulfuric acid on the glass of the flask.

*Source of nitrogen.* Due to the very small amount of growth made by the organism, the amount of nitrogen required is very small: without introducing any nitrogen source into the medium, some growth is obtained, the nitrogen being derived either from the contamination of the other salts, the distilled water, or traces of ammonia in the atmosphere. The best sources of nitrogen are ammonium salts of inorganic acids (particularly sulfate), followed by the ammonium salts of organic acids, after which come the nitrates, asparagin and amino acids. Nitrites, in concentrations used (2 grams per liter) are toxic. Good growth is obtained with pepton, but the amount of sulfur oxidized is less than with the other sources of nitrogen.

*Relation to oxygen.* The organism is strictly aerobic, in view of the fact that it derives the oxygen necessary for the oxidation of sulfur to sulfuric acid from the atmosphere.

*Influence of organic substances.* As pointed out above, glucose does not act injuriously, neither do other organic substances, like pepton. Substances like glycerol, alcohol, mannitol and glucose seem to have a slight favorable effect in the presence of a good nitrogen source. All these substances either act like stimulants or else take part in the structural requirements of the organism.

*Influence of stimulants.* In addition to the pure organic substances, above mentioned, which may stimulate to some extent the growth of the organism, other substances may exert

the same action. A detailed study of the influence of stimulants on the oxidation of sulfur by a pure culture of *Thiobacillus thiooxidans* is presented in table 2.

The medium was buffered with phosphoric acid and mono-potassium-phosphate to a pH of about 3.0. It was distributed

TABLE 2  
*Influence of stimulants on the oxidation of sulfur*

Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub> 0.25 PER CENT	STIMULANT, 0.1 PER CENT	TOTAL SULFATES IN 100 CC. OF MEDIUM MILLIGRAMS	pH	TITRATION*
		<i>mgm.</i>		
+	Control	241.0	3.0	1.0
+		1476.0	1.4	2.8
-		912.5	1.5	2.5
	CaSO <sub>4</sub> (0.25 per cent)	1041.0	1.4	2.7
+	Glucose	991.0	1.5	2.7
-	Glucose	1012.5	1.3	2.6
+	Mannitol	1008.0	1.6	2.5
-	Mannitol	859.0	1.5	2.5
+	Glycerol	858.2	1.6	2.5
-	Glycerol	948.5	1.4	2.6
+	Alcohol	905.0	1.6	2.5
-	Alcohol	994.0	1.4	2.8
+	Soil	1019.5	1.6	2.7
-	Soil	1226.8	1.4	2.6
+	Al <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub>	1394.0	1.4	2.9
-	Al <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub>	989.0	1.4	2.6
+	Thallium nitrate	1133.1	1.5	2.7
-	Thallium nitrate	883.5	1.5	2.5
+	MnSO <sub>4</sub>	1013.4	1.6	2.5
-	MnSO <sub>4</sub>	933.8	1.4	2.5

\*Titration = cubic centimeter of  $\frac{N}{10}$  NaOH necessary to neutralize 1 cc. of culture, with phenolphthalein as an indicator.

in 100 cc. portions in 250 cc. Erlenmeyer flasks containing 1 gram portions of powdered sulfur and the proper amounts of Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>, where present. The flasks were plugged with cotton and sterilized in flowing steam, for thirty minutes, on three consecutive days. The organic substances were sterilized separately, then added to the sterile medium. The flasks were all inoculated with one drop of the same pure culture and incubated



for twelve days. At the end of that period, the pH was determined by the colorimetric method, titration was obtained from the amount, in cubic centimeters, of  $\frac{N}{10}$  NaOH necessary to neutralize 1 cc. of the filtered culture using phenolphthalein as an indicator. The total sulfates were obtained by adding the amounts of soluble and insoluble sulfates: the latter were obtained by digesting the filtered residue in acidulated water and determining the sulfates in an aliquot portion.

In the presence of calcium phosphate, the largest amount of sulfur oxidized by a pure culture of the organism was obtained in medium 1, to which no stimulating agent has been added. In the absence of the tri-calcium phosphate, the amount of sulfur oxidized was appreciably less, oxidation in this case being stimulated by various substances. The most beneficial influence was exerted by the addition of a small amount of soil: this may be due to the introduction, with the soil, of a small amount of the lacking calcium salt or of some vitamine-like substance. The favorable action of the organic substances, aluminum and manganese sulfates, may be of a stimulating nature; however, this beneficial action is only very small and lies within the range of natural variability of the organism.

*Influence of temperature.* The optimum temperature for the activities of *Thiobacillus thiooxidans* n. sp. lies at about 28° to 30°C. Growth and sulfur oxidation are much slower at lower temperatures (18°) and at 37°C. Temperatures of 55°–60°C. are sufficient to kill the organism.

#### THE NATURE OF ACID FORMED AND THE INFLUENCE OF REACTION UPON THE GROWTH OF THIOBACILLUS THIOOXIDANS N. SP.

To get an insight into the true nature of the acid formed, particularly in the presence of tri-calcium phosphate, a series of tubes containing 2 cc. portions of the culture were arranged; measured quantities of  $\frac{N}{1}$  NaOH were added to these, then the volume of the liquid was brought, in all tubes, to 3 cc. by the addition of distilled water. The hydrogen ion concentration of the tubes was then determined, by the colorimetric method. The results are tabulated in table 3 and graphically presented in figure 2.

TABLE 3

*Titration and hydrogen-ion concentration of a 14 day old culture of Thiobacillus thiooxidans n. sp.*

$\frac{N}{1}$ NaOH	pH VALUES	
	No calcium phosphate in the original culture	0.25 per cent of tri-calcium phosphate originally present in the culture
cc.		
0	1.5	1.5
0.02	1.6	1.5
0.04	1.7	1.6
0.06	1.7	1.7
0.08	1.7	1.7
0.10	1.8	1.7
0.12	1.8	1.8
0.14	1.9	1.8
0.16	2.0	1.9
0.18	2.0	1.95
0.20	2.2	2.0
0.22	2.3	2.2
0.24	2.4	2.2
0.26	2.5	2.3
0.28	2.6	2.4
0.30	2.8	3.0
0.32	4.4	3.6
0.34	6.4	4.4
0.36	6.4	5.6
0.38	6.4	6.2
0.40	6.4	6.6
0.42	6.4	6.6
0.44	6.6	6.6
0.46	6.6	6.6
0.48	7.4	6.6
0.50	7.5	6.6
0.52	9.0	7.2
0.54	9.4	7.2
0.56		8.8
0.58		9.6

It will be observed, by glancing at the curves in figure 2, that the hydrogen-ion concentration slowly decreases, as manifested by a slow increase in the pH values, with the addition of alkali, till the pH reaches 2.8, then there is a sudden drop in the curve, to pH 6.4, when the curve again becomes slanting, followed by a second drop. This gives the buffer effect of the cultures; the buffer action is more pronounced in the presence of tri-calcium phosphate, which increases the phosphate content of the medium.

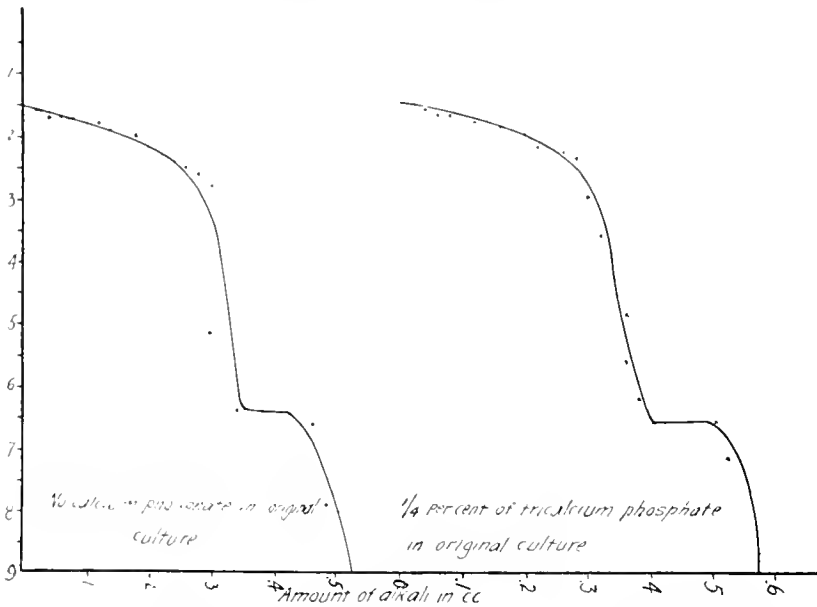


FIG. 2. TITRATION CURVES OF THE CULTURE OF *Thibacillus thiooxidans* N. SP.

The sulfur is oxidized into sulfuric acid; this acid acts upon the tri-calcium phosphate transforming it first into the di-calcium salt, then the mono-calcium salt, and finally into phosphoric acid, while the calcium is precipitated as calcium sulfate; further oxidation of sulfur results in the production of free sulfuric acid.

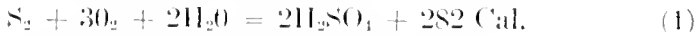
As to the influence of initial reaction upon growth, we find that a reaction having a hydrogen-ion concentration equivalent to a pH of 2.0-2.8 is the most favorable for the growth of the

organism. Reactions more acid than 2.0 easily become injurious, although the organisms still continue to live at even as low a reaction as a hydrogen-ion concentration of  $\text{pH} = 0.6$ , while the medium titrates 0.8 normal acid (with phenolphthalein as indicator, using  $\frac{N}{10}$  NaOH: the culture being grown on medium 1). Reactions ranging in pH from 4.0 to 6.0 are less favorable. Growth is slower to start, but once the reaction, through a slow oxidation of the sulfur, has reached a pH of about 3.0, the growth becomes more rapid. Reactions equivalent to pH 6.0 and above are unfavorable for growth. When a culture, at a pH 0.8 to 1.6 (these being the limits tested), is filtered free from any unoxidized sulfur, then stoppered and allowed to stand, the liquid is found to clear up, after a period of time, and the bacteria are agglutinated with the formation of flaky masses at the bottom of the containers. The rapidity of agglutination depends on the reaction of the culture, the more acid cultures agglutinating more rapidly than the less acid ones: at a pH = 0.8, agglutination took place in four to five days, while at pH = 1.5, it took more than two weeks for this phenomenon to appear. It is interesting to note that this phenomenon was never observed in the unfiltered culture, i.e., in the presence of unoxidized sulfur, even if the cultures were kept at pH 0.6 to 0.8 for a long time.

*Neutralizing agents.* The acid formed rapidly changes the hydrogen-ion concentration of the medium and growth almost ceases. To obviate a rapid change in reaction by the acid produced from the oxidation of the sulfur, neutralizing agents are to be used. These should be of such a nature as not to make the medium alkaline or tend to change the reaction rapidly: this eliminates, therefore, the use of carbonates and soluble oxides, like CaO. The best substances are buffers, like phosphates, but to keep the reaction above a very high acidity by means of soluble phosphates, high concentrations have to be used, which will exert an unfavorable physical effect upon the organism. Solid salts, insoluble in water which, on dissolving by the action of the acid, will give a soluble substance and an insoluble residue, are best for this purpose.  $\text{CaCO}_3$  and  $\text{MgCO}_3$  can be used, but

these go rapidly into solution by the action of the acid, thus tending to change the reaction towards alkaline.  $\text{Ca}_3(\text{PO}_4)_2$  offers the best material for the purpose, because, on dissolving, it gives an acid salt and an insoluble residue ( $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ ).

*Mechanism of sulfur oxidation.* The sulfur is oxidized, according to the following reaction:



In the presence of tri-calcium phosphate:



The energy liberated in the oxidation of sulfur is used by the organism for its activities. The acid formed interacts with the neutralizing agents of the medium, giving first mono-calcium phosphate, at a pH of about 2.8-3.0, then phosphoric acid. So that, at a condition of equilibrium, we have a mixture of phosphoric and sulfuric acids, and the calcium salts of these acids, the condition of equilibrium depending on the stage of oxidation.

*Taxonomic considerations.* The first paper of this series contains a study of the five groups of sulfur bacteria. The organism described in this paper, *Thiobacillus thiooxidans* n. sp., is placed in a fifth group, which includes members morphologically related to the members of the fourth group, but which are distinctly different physiologically.

Group four includes colorless sulfur bacteria which do not accumulate sulfur within their cells, but which produce an abundance of sulfur (from  $\text{H}_2\text{S}$  and thiosulfates) outside of their cells. This group of bacteria is the one closely related to the organism studied in this paper and will, therefore, be discussed in greater detail. Group four is represented by two bacteria, *Thiobacillus thioparus* (Nathanson) Beijerinck and *Thiobacillus denitrificans* Beijerinck. Group five, which is so far represented only by *Thiobacillus thiooxidans* n. sp., will include colorless sulfur-oxidizing bacteria which do not accumulate sulfur either



FIG. 3. *Thiobacillus thiooxidans*, n. sp. ( $\times 1500$ ). CULTURE GROWN IN INORGANIC MEDIUM

Stained with aqueous-alcoholic solution of gentian violet

within or without their cells, which are very small in size (a micron or less in length and 0.5 micron in diameter) and which oxidize sulfur rapidly to sulfuric acid with a very acid reaction.

*Thiobacillus thioparus* was demonstrated by Nathanson in sea water and by Beijerinck in canal water. It was isolated on a medium containing sodium thiosulfate as a source of sulfur, in addition to minerals and ammonium chloride (0.01 per cent) and sodium bicarbonate as a source of carbon. In two to three days, the surface of the medium became covered with free sulfur filled with bacteria. This organism is 3 by  $0.5\mu$ , not forming any spores, is very motile and very sensitive, dying out on the plate in a week. The thiosulfates can be replaced by CaS,  $H_2S$  and elementary sulfur.

Jacobsen dissolved the sulfur in sodium sulfide and precipitated with dilute hydrochloric acid, washed and dried, then added to a medium containing 100 parts of water, 0.05  $K_2HPO_4$ , 0.05  $NH_4Cl$ , 0.02  $MgCl_2$ , 2 of  $CaCO_3$  or  $MgCO_3$  and a trace of  $FeCl_3$  (3 parts of  $NaCl$  were used in the case of the organism isolated from sea water). The cultures were incubated at  $30^\circ C$ . The organism was found to form a film on the surface of the culture, with sulfur granules surrounding the cells; at the end, instead of sulfur, only a slimy bacterial mass was found to remain. Traces of hydrogen sulfide were always found. Pure cultures of the organism were obtained on agar plates, using 0.5 per cent of sodium thiosulfate and some  $CaCO_3$ . The carbon dioxide is obtained from carbonates, no growth being obtained and no sulfuric acid produced in the absence of carbonates. The organism is autotrophic since it does not require any organic matter for its development; it is sometimes motile and sometimes non-motile.

*Thiobacillus denitrificans* was isolated by Beijerinck by adding to 100 parts of canal water, 10 parts of powdered sulfur, 0.05  $KNO_3$ , 0.02  $Na_2CO_3$ , 2  $CaCO_3$ , 0.02  $K_2HPO_4$  and 0.01 part of  $MgCl_2$ , and incubating the medium at  $30^\circ C$ . The sulfur was oxidized and growth was accompanied by a reduction of the nitrate to atmospheric nitrogen. The organism was isolated on agar plates and was found to be a motile, short rod, hardly distinguishable

morphologically from the *Thiobacillus thioparus*. Both organisms use carbonates and bicarbonates as sources of carbon and rapidly lose, on the plate, their ability to grow.

The following table gives the salient features of organisms belonging to groups 4 and 5.

*Autotrophy.* *Thiobacillus thiooxidans* belongs to the autotrophic bacteria which derive their energy from inorganic substances,

TABLE 4  
*Salient features of sulfur oxidizing bacteria, not accumulating sulfur within their cells*

	TH. THIOPARUS (NATHANSON) BEIJERINCK	TH. DENITRIFICANS BEIJERINCK	TH. THIOOXIDANS N. SP.
Energy.....	H <sub>2</sub> S, thiosulfate, sulfur	H <sub>2</sub> S, thiosulfate, sulfur	Sulfur, thiosulfate
Size.....	3 by 0.5 $\mu$	3 by 0.5 $\mu$	1 by 0.5 $\mu$
Accumulation of sulfur outside the cell.....	+++	+++	None
Pellicle formation.....	+	+	None
Carbon sources.....	Carbonates, bicarbonates	Carbonates, bicarbonates	CO <sub>2</sub> from atmosphere
Aerobism.....	Aerobic	Anaerobic	Aerobic
Growth on agar media.....	+	+	-
Motility.....	+	+	$\pm$
Acid accumulation.....	Active	?	Very strong, pH goes down to 0.6

and its carbon from the CO<sub>2</sub> of the atmosphere. This bacterium, which can derive its carbon from the CO<sub>2</sub> of the atmosphere, its energy from inorganic sulfur, its nitrogen from ammonium sulfate and other inorganic salts and whose mineral need is very small, was probably among the very first to start life on our planet. The sulfuric acid formed interacted with the insoluble silicates, phosphates, carbonates, etc., thus helping to break down the original rock and allowing the life of other organisms to follow. This organism or, perhaps group of organisms, together with the nitrifying bacteria may thus have formed the initial step in the organic world, manufacturing organic materials for other forms of life to follow.



## SUMMARY

1. *Thiobacillus thiooxidans* n. sp. was isolated from composts of soil, sulfur and rock phosphate, by the use of inorganic media.

2. It oxidizes elementary sulfur to sulfuric acid, derives the necessary carbon from the CO<sub>2</sub> of the atmosphere and its nitrogen need from inorganic nitrogen salts.

3. It is responsible for the oxidation of sulfur in the soil and when soil is composted with sulfur or with sulfur and rock phosphate.

4. The sulfuric acid produced from the oxidation of sulfur by *Thiobacillus thiooxidans* n. sp. transforms tri-calcium phosphate into soluble phosphates and finally into phosphoric acid.

5. *Thiobacillus thiooxidans* n. sp. produces more acid, from oxidation of sulfur, and continues to live in a more acid medium, than any other living organism yet reported, the hydrogen-ion concentration of the medium increasing to a pH 0.6 and less.

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# THE PRODUCTION OF PINK SAUERKRAUT BY YEASTS<sup>1</sup>

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Sauerkraut, or sour cabbage as it is sometimes called, is obtained by the acid fermentation of cabbage. The process of fermentation and manufacture is simple and the resulting product is greatly relished by many people. Some idea of the importance of this method of preserving cabbage may be seen from a glance at the sauerkraut industry in Wisconsin. In this state alone more than 36,000,000 pounds of sauerkraut are manufactured annually, in addition to that prepared in small quantities in innumerable households.

Normal sauerkraut has a distinctly acid reaction and a faint pleasant aroma. The shredded cabbage after it has turned into kraut loses some of its toughness, but should still retain a comparatively firm texture; the white color tends to lose its opaqueness and the cabbage becomes slightly translucent. Kraut with a strong odor and soft texture is of poor quality.

The preservation of cabbage in the form of sauerkraut is generally a result of natural fermentation. Clean white cabbage is cut into shreds, salt is added, and the entire mass packed into a vat and heavy weights are placed on top. In a few hours fermentation begins and the sugars of the cabbage are rapidly converted into lactic acid, acetic acid, alcohol, and small amounts of other products. Although many kinds of microorganisms may be found in the juice of the kraut, the lactic acid bacteria are the most important.

<sup>1</sup> Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

In recent years, some of the canning companies have found it difficult always to secure a sauerkraut of good flavor, texture, and color. One of the difficulties encountered, has been the occurrence of a sauerkraut with a well defined pink or salmon-pink color. Although not unfit for food, this pink colored sauerkraut is an undesirable product as it must be sold at a price lower than that obtained for white kraut.

From a review of the literature, it seems probable that microorganisms are involved in the formation of the pigment. In 1904 Butjagin, and a year later, Wehmer called attention to the occurrence of pink producing organisms in sauerkraut. Henneberg in 1916 reported that the addition of 1.2 per cent of lactic acid to cabbage resulted in the production of a reddish colored kraut. The presence of large numbers of yeasts in sauerkraut, and in a few cases of pink yeasts, has been reported by various investigators. The distribution of these pink yeasts in nature and the factors that influence pigment formation have been the source of much study. Grösbusch (1915) isolated from apples a colorless torula which under certain conditions formed a deep red pigment. Some of the conditions which he found favorable for pigment production, were low sugar content, certain kinds of sugars, and a slightly acid reaction. Beijerinck (1919) described a yeast producing a colorless substance which became a deep red pigment in the presence of oxygen and iron salts. A complete discussion of the literature of the pink yeasts will be found in the papers of Will (1907; 1912), Pringsheim and Bilewsky (1911). In view of the occurrence in practise of the colored sauerkraut it becomes a matter of some importance to discover the cause, and if possible, the remedy for this undesirable type of fermentation.

#### EXPERIMENTAL

Large samples of normal sauerkraut and pink sauerkraut were secured from one of the canning companies and analyzed. This kraut was six weeks old and judged proper for canning. The comparative chemical and bacteriological analyses are given below:

*Analysis of sauerkraut*

	NORMAL.	PINK
<i>Chemical:</i>		
1. Per cent of water in kraut.....	90.6	88.0
2. Total titratable acid in 100 cc. juice..	140.0 cc. 0.1N	147.5 cc. 0.1N
3. Volatile acid as acetic in 100 cc. juice..	0.247 gram	0.255 gram
4. Non-volatile acid as lactic in 100 cc. juice.....	1.026 grams	1.426 grams
5. Alcohol as ethyl.....	0.727 gram	0.978 gram
<i>Bacteriological:</i>		
Number of microorganisms in 1 cc. juice...	3,600,000	91,000,000

Chemical analysis failed to show any striking difference in composition between the normal and the pink sauerkraut. Later analyses of other samples indicate that the figures given above are fairly representative of the two kinds of sauerkraut. The results of the bacteriological analyses are of more significance and show that the colored kraut is much richer in microorganisms. By means of direct microscopic mounts from the normal kraut it was found that there was a preponderance of rod-formed bacteria while the pink kraut contained yeast cells almost exclusively. The enormous number of yeast cells in the juice of pink kraut suggested that these organisms might be the cause of the pink pigment. Prompted by the fact that yeasts are usually present in high numbers in pink sauerkraut a great number of dilution plates were poured. Almost without exception the colonies on these plates consisted of yeasts<sup>2</sup> but rarely was any pigment developed.

Many samples of pink sauerkraut were plated and from well isolated colonies transfers were made to glucose yeast-water agar slants. In general these yeasts from pink kraut showed little if any color on agar slants. A few transfers gave a pale pink color. From a large number of cultures three pink colored colonies were selected for further study, numbered 24-1, 85-1, and 95-6. These strains show a difference in color; 24.1 and

<sup>2</sup> The term yeasts in this paper is used to designate the true *saccharomycetes* and also those which do not form ascospores, the *torulae*. It is probable from the results of previous workers that these pink yeasts are properly termed *torulae*.

95.6 are a salmon shade of pink and 85.1 is a magenta shade. In form these organisms vary from round to oval, but elongated cells were the most abundant.

#### *Factors that influence color*

*Oxygen.* The influence of oxygen on the production of pigment by these yeasts was determined by growing the cultures in the air, and in a desiccator where only a limited supply of air was available. In the presence of air the growth and pigment production was good while in its absence a fair growth and no color was obtained. When these colorless tubes were exposed to the atmosphere a salmon-pink color developed rapidly usually within one to two days. The influence of oxygen was also noted when kraut was exposed to the air, e.g., pale pink kraut turned a deeper color after a few minutes exposure.

*Reaction.* The reaction of the medium for cultivating these organisms was varied between the pH values of 6.5 and 7.2. Pigment was formed in all cases and approximately to the same extent. At a reaction of 5.5 the growth was not so rapid, but the color was somewhat more brilliant.

*Temperature.* The influence of this agent on color formation by these yeasts was studied at 18°, 22°, 28°, and 37°C. Only a very scanty growth was noted at 37°C, a profuse growth at 22° and 28°C, and a fair growth at 18°C. The deepest pigment was found in the tube cultures at 22°C. and next in intensity of color, at 28°C. Although the higher temperature of 28°C. resulted in a profuse growth the pink color was not nearly so noticeable as at lower temperatures. Apparently about 22°C. or 71°F. gave the most marked color.

*Iron and manganese salts.* The influence of iron citrate, iron ammonium citrate, iron lactate, iron sulphates, and manganese sulphate, on pigment formation was studied. A decided difference in behavior of the various strains of yeast towards the iron and manganese compounds was noted. Strain S5-1 responded far more to the iron salts than either strain 24-1, or 95-6. Of the various compounds, iron lactate proved the best stimulant for pigment production. The other compounds, e.g.,

iron citrate and iron ammonium citrate also favored pigment production. The manganese salts apparently promoted growth but did not bring about an increase in the pigment formation.

*Sugars.* In order to overcome as much as possible the breaking down of the sugars from the high heat, concentrated solutions of sugars in water were sterilized and added to the culture medium when cool. The yeast medium contained the following constituents:

Ammonium sulphate.....	3.0 grams
Asparagin.....	1.5 grams
Dibasic potassium phosphate.....	2.0 grams
Calcium chloride.....	0.25 gram
Magnesium sulphate.....	0.25 gram
Sugar.....	20.0 grams
Agar.....	15.0 grams
Water.....	1000.0 cc.

Three sugars were studied; xylose, glucose and maltose in 2 per cent solutions. Each strain of yeast was grown in triplicate on agar slants, containing each sugar, and these cultures were incubated at 20° to 22°C. At regular intervals, usually of 1 week each, these cultures were examined for rate of growth and pigment production.

All three strains showed by far the most rapid growth in the glucose medium, with maltose next in order, and xylose last. The decided difference between growth and pigment formation is brought out in a striking manner from the results of this test. Without exception the easily fermentable sugar, glucose, gave a profuse growth and only a trace of pigment. Somewhat similar results were obtained with maltose although a pale pink was noted. In the presence of xylose these yeasts grew slowly but produced a decided pink color. From these results it is clear that the sugar, xylose, which is fermented only with difficulty is the best one of the three for pigment production. After thirty days the glucose cultures entirely lost their color while the other cultures became a deeper pink. Our results agree with those of Grösbusch, who found that the best color was obtained in the case of the non-fermentable sugars, arabinose and raffinose.

TABLE 1  
Effect of sodium chloride on pigment production

NUMBER	TIME	SODIUM CHLORIDE		XYLOSE		GLUCOSE		MALTOSE	
		per cent	Growth	Color	Growth	Color	Growth	Color	
Cultures 24-1									
1	8	None	Good	Pink	Profuse	Pink	Profuse	Pink	Pink
2	8	2	Fair	Pink	Profuse	Pink	Profuse	Pink	Pink
3	8	4	Trace	Pale pink	Fair	Pink	Moderate	Pink	Pink
4	30	None	Profuse	Pink	Profuse	Pale pink†	Profuse	Pink	Pink
5	30	2	Profuse	Pink	Profuse	Pink	Profuse	Brilliant pink	Brilliant pink
6	30	4	Moderate	Brilliant pink	Profuse	Pink	Profuse	Brilliant pink	Brilliant pink
Cultures 85-1									
7	8	None	Scanty	No color	Scanty	Pale pink	Scanty	No color	No color
8	8	2	Scanty	No color	Scanty	Pale pink	Scanty	No color	No color
9	8	4	Scanty	No color	Scanty	Pale pink	Scanty	No color	No color
10	30	None	Moderate	Pink*	Fair	Pale pink	Slight	Pale pink	Pale pink
11	30	2	Moderate	Pink	Fair	Pink	Slight	Pink	Pink
12	30	4	Moderate	Pink	Fair	Pink	Slight	Pink	Pink
Cultures 95-6									
13	8	None	Scanty	Pink	Scanty	No color	Scanty	Pink	Pink
14	8	2	Scanty	Pale pink	Scanty	No color	Scanty	Pale pink	Pale pink
15	8	4	Scanty	Pale pink	Scanty	No color	Scanty	Pale pink	Pale pink
16	30	None	Fair	Pink	Scanty	No color	Fair	Pale pink	Pale pink
17	30	2	Fair	Pink	Scanty	No color	Fair	Pink	Pink
18	30	4	Fair	Pale pink	Scanty	No color	Fair	Pink	Pink

\* Deeper pink than any other sugar.

† Beginning to lose color.



*Sodium chloride.* The influence of salt on pigment production naturally suggested itself since it has been reported by many experienced makers of sauerkraut, that high concentrations of salt caused the reddening of the kraut. Sodium chloride in concentration of 2 and 4 per cent was added to triplicate tubes of the glucose, maltose and xylose agar media. These tubes were inoculated with each yeast. 2.5 per cent salt is the approximate amount used in the manufacture of commercial kraut. In table 1 are given a summary of the results of these tests. The cultures plus salt developed much more slowly at first than those without salt, but after two weeks this retardation was not so noticeable. In the glucose series, culture 24-1, no salt, the pink color began to fade to a pale pink at the end of two weeks, while in the presence of salt a brilliant pink pigment persisted. These cultures were kept for 6 weeks but without any loss of color, save in the no-salt group. Instead of a loss, the older cultures of the salt group showed a greater amount of pigment and a deeper color. Somewhat similar results were secured with the other strains of yeasts although the intensifying effect of salt was not so noticeable. It seems safe to conclude that sodium chloride even in large amounts exerts a favorable effect on pigment production. This favorable influence is not noticeable until the cultures are several weeks old.

#### PRODUCTION OF PINK SAUERKRAUT BY INOCULATION WITH YEASTS

In large glass percolators of 2 liter capacity 1000 grams of cut cabbage were packed. The outside leaves and core of the raw cabbage were removed, it was cut on a small shredder and salt was added. Part of the cabbage was inoculated with cultures of the yeast isolated from pink kraut. The entire mass was packed into the percolator and weighted down with a bottle of sand or mercury, that weighed 1 kilo and which fitted closely in the top of the percolator. The lower end of the percolator was fitted with glass wool, and below this was inserted a one hole rubber stopper fitted with a glass tube. Through this glass tube, which was sealed at one end with a rubber tube and screw clamp,

samples of the fermenting liquid were removed from time to time and the total titratable acidity measured. For comparison, samples were also taken from the top of the percolators and titrated. No very decided difference was noted in titration figures from the different parts of the same container.

Since it is highly important to note the color changes during fermentation, percolators were found especially suitable for this study.

The plan of this experiment follows:

1. 2 per cent salt no inoculation,..... Control
2. 2 per cent salt no inoculation,..... Control
3. 2 per cent salt inoculated with yeast.....24-1
4. 4 per cent salt inoculated with yeast.....24-1
5. 2 per cent salt inoculated with yeast.....85-1

After four days the cabbage in percolators, numbers 3, 4, and 5 showed numerous pink colored spots and two days later the the entire mass became pink throughout. The pink pigment of numbers 3 and 4 especially increased with age, until at the end of two weeks the plant tissue was a deep red or a purplish red color. This increase in color as the kraut aged, was not noted in the cabbage inoculated with 85-1. When four weeks old the kraut was removed and examined.

Numbers 1 and 2 were sour, rather tough and judged of fair quality. Direct microscopic mounts showed a preponderance of bacteria.

Numbers 3 and 4 were bitter with an unpleasant flavor. Microscopic mounts showed an almost equal number of yeasts and bacteria.

Total acid determinations, made at two-day intervals failed to bring out any very striking differences; in general the containers with yeasts showed less acid at the end of the experiment than the untreated controls. The total titratable acidity at the time of opening is given below:

	<i>0.1 N acid in 100 cc. of juice cc.</i>
1. Control.....	218.0
2. Control.....	162.0
3. Inoculated with 24-1.....	73 0
4. Inoculated with 24-1.....	144 0
5. Inoculated with 85-1.....	179.0

EFFECT OF SODIUM CHLORIDE AND OF TEMPERATURE ON THE PRODUCTION OF PINK SAUERKRAUT

Attention has been called to the statement of sauerkraut manufacturers that salt favors the pink color. Laboratory tests with pure cultures of pink yeasts also indicate the favorable

TABLE 2

*The influence of sodium chloride and temperature on the production of pink sauerkraut*

AGE OF KRAUT	SODIUM CHLO- RIDE	TEMPERATURE IN CENTIGRADE		
		28°	21°	16°
<i>days</i>	<i>per cent</i>			
7	2.5	A trace of pink	No pink color	No pink color
7	3.5	A trace of pink	No pink color	No pink color
7	4.5	Pink at bottom	No pink color	No pink color
7	5.5	Brilliant pink throughout	No pink color	No pink color
10	2.5	Spots of pale pink	No pink color	No pink color
10	3.5	More pink than above	No pink color	No pink color
10	4.5	Pink at bottom	No pink color	No pink color
10	5.5	Brilliant pink throughout	No pink color	No pink color
20	2.5	Pale pink	No pink color	No pink color
20	3.5	Pink	Pale pink	No pink color
20	4.5	Brilliant pink throughout	Pink spots	No pink color
20	5.5	Brilliant pink throughout	Pink throughout	Pink spots

influence of this chemical on color production. To see if the raw cabbage without inoculation, but high in salt, would undergo a fermentation which produced a pink color, 12 large percolators of cabbage were treated as outlined in the table below and incubated at three temperatures, 16°, 21°, and 28°C. The procedure

was the same as in the previous experiment. Table 2 gives the general plan and results of this test. The data recorded in the table show: first, the close relation between amount of sodium chloride and formation of pink sauerkraut; second, the decided influence of temperature on color. These two points are so clearly brought out in the table that a detailed explanation is not needed. The findings are in accord with the observations of kraut manufacturers; namely, that high salt concentration may cause pink sauerkraut.

TABLE 3  
*Effect of sodium chloride on the production of acids by lactic acid bacteria;  
calculated for 100 cc. of culture*

NUMBER	MEDIUM	TIME AFTER	SODIUM CHLORIDE	0.1N ACID
		<i>days</i>	<i>grams</i>	<i>cc.</i>
1	Cabbage juice	3	None	70.4
2	Cabbage juice	3	1	55.6
3	Cabbage juice	3	2	49.8
4	Cabbage juice	3	3	32.2
5	Cabbage juice	3	4	1.8
6	Cabbage juice	3	5	1.2
7	Cabbage juice	3	6	0.0

An explanation for this condition may be found from a study of pure cultures of yeasts and bacteria common to sauerkraut. Yeasts can live in the presence of high concentrations of salt while the bacteria commonly associated with kraut are not so resistant. Support for this statement is given in the figures of table 3. Here the influence of varying amounts of sodium chloride on a pure culture of lactic acid bacteria was studied. This lactic acid organism was isolated from normal sauerkraut. About 4 per cent of salt is the critical concentration for this organism.

Orla-Jensen (1919) has carried out an extensive study of the influence of sodium chloride on acid production by various types of lactic acid bacteria. He found that the common forms of lactic acid bacteria, the *Bacterium lactis-acidi*, *Lactobacillus bulgaricus*, and other types are slightly retarded by 2.5 per cent of salt. On the other hand, certain of the rod forms of lactic

acid bacteria isolated from plant tissue are not retarded in the least by this concentration of salt. In the presence of larger amounts of salt (5.5 per cent), all of the rod forms of lactic bacteria are injured. A concentration of not more than 2 to 4 per cent of salt would not seriously retard the growth of the yeast and would favor its production of pigment.

#### EFFECT OF HIGH ACID FORMING BACTERIA ON THE PRODUCTION OF PINK SAUERKRAUT

The use of bacteria which would ferment the sugars of the cabbage juice rapidly naturally suggests itself as a means of combating the growth of pink yeasts. If great numbers of acid producing bacteria are seeded in the raw cabbage then it is possible that they will so dominate the fermentation that the wild yeasts will not be able to exert any well defined effect on the kraut. The fallacy of this assumption, however, is clearly seen from the results of table 4. As in the preceding tests large percolators with 1000 grams each of raw cabbage were used. The data include the results of four separate tests carried out at different times. In every case before the end of the fermentation period the cabbage to which acid was added or which was inoculated with high acid-producing bacteria turned pink. Apparently the high acid production which followed inoculation favored the growth of pink yeasts. Direct microscopic mounts made from this pink sauerkraut furnished support for this statement. When opened, the inoculated kraut showed great numbers of yeasts. Some idea of the rate of acid formation may be gained from the figures in this table. In the early stages of fermentation, without exception, the kraut seeded with aciduric bacteria showed a much greater quantity of acid than the control kraut. This difference is especially noticeable in the titration figures of the first four or five days. After eight days, conditions changed and before the end of the fermentation the control krauts were far more acid than the inoculated krauts. Culture 124-1 which is a high acid former from pentoses and other sugars is especially favorable for the development of the pink yeasts. On the other hand the non-pentose fermenter *Bact. lactis-acidi* which

produces acid more slowly than Culture 124-1, does not favor the growth of the pink yeasts. Perhaps the beneficial effect of inoculation on pigment production is due to the rapid destruction of the easily fermentable sugars and to the change in reaction. This combination of conditions no doubt reduces greatly the complex of the bacterial life and thus favors the development of the wild yeasts.

TABLE 4

*The relation of acid forming bacteria and pink yeast to the production of pink sauerkraut*

NUMBER	TREATMENT	0.1N ACID IN 100 CC. OF JUICE				NOTES	
		Age in days				Quality	Appearance
		2	4	8	20		
cc.	cc.	cc.	cc.				
1	Control.....	28	79	195	192	Fair	White
2	<i>Lactob. bulgaricus</i> .....	83	136	156	155	Poor	Pink
3	Control.....	23	99	165	218	Raw	Trace of pink
4	Culture 52-7.....	92	120	122	169	Fair	Pale pink
5	Culture 124-1.....	111	150	168	152	Raw	Brilliant pink
6	Culture 124-1.....	114	150		161	Raw	Pink
7	Control.....	25	79	121	197	Good	White
8	Culture 124-1.....	95	131	145	153	Poor	Pink
9	Culture 124-1.....	84	135	140	151	Poor	Pink
10	Control + acids*.....	110	121	125	192	Poor	Deep pink
11	Culture 85-1.....	23		43	114	Fair	White
12	Culture 85-1 + acids*..	110		130	173	Raw	Deep pink
13	Culture 85-1 and 124-1.	39		115	118	Poor	Pink
14	Culture 24-1 and <i>Bact.</i> <i>lactis acidii</i> .....	31		96	142	Good	White

\* Acetic and lactic acids equivalent to 109 cc. 0.1N per 100 cc. of juice.

The stimulating effect of acids on pigment production is further indicated by the results obtained in containers Nos. 10 and 12 where acetic and lactic acids were added to uninoculated cabbage and to cabbage inoculated with yeasts. The quantity of acid added was approximately that found in a moderately ripened kraut. Pigment was observed after seven days which is about the earliest appearance of color in any of the experiments.

## SUMMARY

The pink or red color of sauerkraut is due to the growth of certain yeasts or torulae. Although these organisms are commonly found in great numbers in kraut, they frequently fail to show any pigment. The production of pigment is not a fixed characteristic but depends upon many factors. The following conditions influence the formation of color: kind of sugar, amount of sugar, amount of sodium chloride, reaction, temperature, and oxygen supply. The chief factors in the production of pink sauerkraut are high temperature, high salt concentration and high acid content. When cabbage is allowed to ferment at a temperature of 20°C. or above a pink pigment is frequently noted. Almost without exception, cabbage fermented in the presence of large amounts of sodium chloride, 3 per cent or more, showed a decided pink or red color. High acid producing bacteria as well as the direct addition of acids to cabbage favor the development of pink kraut.

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STUDIES ON THE BIOLOGY OF LACTIC ACID  
BACTERIA: A SUMMARY OF PERSONAL  
INVESTIGATIONS<sup>1</sup>

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In the following pages are summarized the most important results of investigations which I have pursued during the recent war period upon the biological characteristics of the lactic acid bacteria.

ACIDO-PROTEOLYTIC PROPERTIES

One of the most important characteristics of many lactic acid bacteria is that of possessing acido-proteolytic properties. After the original discovery of this property I have often asserted that this activity must take place in natural milk of acid reaction, but that it is not observed in milk which has received the addition of chalk or other substances, since these additions not only alter the composition of the milk but also so alter its adaptability for the growth of the organisms concerned that the natural functions of the latter are no more exhibited and thus the results obtained in the study are no longer applicable directly to normal conditions such as occur in the cheese industry.

The detection of this acido-proteolytic property is very simple and indeed requires no chemical manipulations. Just as the liquefaction of gelatin indicates proteolytic properties, the casein cleaving properties of these organisms are indicated by the

<sup>1</sup>The investigations covered by this summary have been published in the transactions of the Reale Accademia dei Lincei of Rome and the transactions of the Reale Istituto Lombardo di Scienze e Lettere of Milan during the years 1914-1920.

liquefaction of the casein coagulum. I have described, as organisms possessing acido-rennin properties, cocci from the udder of the cow, and bacilli from cheese and fermented milk beverages such as Yoghurt and Gioddu. The inception of casein breakdown is dependent upon various circumstances; chief among these, and of paramount importance, is *temperature*.

In 1897 I demonstrated that while high temperatures are appropriate to the process of lactose fermentation, lower temperatures are more appropriate to the cleavage of casein, and in several additional publications I was able to verify my findings. Finally in 1915 I presented analytical data bearing upon the behavior of lactococci and lactobacilli at temperatures ranging between 25° and 35°C. and 15° to 20°C. Many lactic acid forming bacteria which failed to show lacto-proteolytic properties when cultivated at the higher temperatures were found to possess these properties when cultivated at the lower temperatures, *i.e.*, such temperatures as prevail during the process of cheese ripening.

Another condition of great importance for the furthering of the lacto-proteolytic activities is the *composition of the medium*. Already in 1902 I had advanced evidence as to the influence of the nature of the protein upon the activities of the organisms, indicating that while among the udder cocci some dissolved casein as well as gelatin, others only dissolved casein while again others were found that acted only on gelatin.

Another factor of great importance was found to lie in the *quality of the milk* itself, which presents great fluctuations the causes of which are to be found in the changes it undergoes previous to reaching the laboratory (besides the influence exerted by the race of the animals furnishing the milk, their physiological state, their feeding régime, etc.) or in the laboratory itself. In addition, in 1915, I was able to show the noxious influence exerted by the peptonized constituents of the milk derived from the casein cleavage. These constituents are often to be found in milk, especially in market milk, and are due to the great development of organisms before sterilization.

Another factor of great importance, and indeed of an essential character in the behavior of milk, is to be found in the *method of*

*sterilization* previous to use. Milk sterilized in the autoclave acquires a brownish tinge, indicative of a change of state, which makes it unfit for the demonstration of the proteolytic activities of the lactic acid bacteria. For such a study milk should be utilized that has been sterilized at a moderate heat so that its white color has been preserved. By using this white-sterile milk I was in a position to detect casein cleaving properties in some of the lactic acid bacteria which in brown-sterile milk failed to digest this compound. Some of the so-called propionic acid bacteria behaved similarly.

Undoubtedly, in this factor of appropriate sterilization is to be found the reason why many authors claim for the lactic acid bacteria negative or only negligible casein-acido-dissolving properties, and therefore contrary to my opinion have ascribed to them no rôle in the ripening of cheese.

#### BACTERIAL FLORA OF THE UDDER

The importance which the acid forming organisms seem to bear, both from the standpoint of hygiene and in the dairy, gave me occasion for a deep study. It is to be emphasized nevertheless that investigations seem to point to the fact that from the hygienic and sanitary standpoint it is not only the cleanliness of the stable and of the cow that are to be considered, but also other internal and external factors. A fact of great importance in this connection is, that apparently sound cows may harbor in their udder for longer or shorter periods of time bacteria (cocci as well as bacilli) which sometimes prove beneficial and other times noxious both from the hygienic as well as from the dairying standpoint. Therefore a selection of milch cows on the basis of this udder flora has been proved useful by me and to this I have given new support by carrying out fermentation tests as a means of judging of the quality of the milk, especially of such milk as is used for direct consumption by children and invalids.

#### HEAT RESISTANCE

It is generally assumed that an exposure of one quarter of an hour at 60° to 80°C. is sufficient to destroy the non-spore-forming

lactic bacteria. On the contrary I have discovered that the lethal temperature may reach 100°C. and sometimes still higher values.

My experiments have shown that this happens through the formation of a sheath of coagulated casein in immediate contact with the acid-rennin forming organisms. These findings allowed me to explain the apparent failures in milk sterilization, and they furnish the basis for a betterment of the processes in use for industrial milk sterilization.

#### VISCOSITY IN YOUNG CULTURES

Many lactic acid forming organisms are capable of inducing ropiness in milk only during the early phases of incubation. The property of making milk ropy, although recognized by various investigators for the lactic acid bacteria, has not generally been considered as a constant and essential character of this group of organisms. In 1912 I first described a lactic acid bacterium which constantly induced viscosity in milk, but only on the inception of acidification and before the inception of coagulation.

This organism at first induces ropiness, but the increase in acidity of the medium and the increase in the firmness of the coagulum soon obliterate this characteristic result of the growth. When cultivated at a not too high temperature and on white-sterile milk several other lactic organisms proved themselves, in my hands, to possess this property. The reason why many investigators disagree as to the constancy of this rope-producing property in many of the lactic acid bacteria is to be searched for in its transitory manifestation.

#### SPORE-PRODUCING LACTIC ACID BACTERIA

In 1904 I studied a spore-forming bacillus from cheese and described it under the name of *Bacillus acidificans-presamigenes-casei*, on account of its acid-rennet and acido-proteolytic characters and found it related to the subtilis or thyrotrix groups. In 1906 I found in silage another similar form and described and pictured it. Additional investigations have authorized me in the assertion that this type is rather widely disseminated in milk and dairy products and is therefore worthy of attentive study.

APPLICATION OF LACTIC ACID BACTERIA IN THE CHEESE INDUSTRY  
AND IN THE PREPARATION OF ACID SILAGE

In 1906 I first drew attention to the advantage to be derived from the utilization of the lactic acid bacteria in cheese manufacture, and in 1907 I recommended their utilization in the preparation of silage. Although from the beginning of my investigations I was fortunate in the selection of the appropriate types, so that their practical application now dates back fifteen years, my study was continued upon the various types which from time to time have attracted my attention. The result of these investigations is that several species, or varieties, of lactic acid bacteria can be utilized for the purpose, but that nevertheless it is not a matter of indifference, for the preservation of the character of determined cheese quality, which type has been used.

Two advantages are to be derived from the utilization of the lactic acid bacteria in cheese manufacture, *i.e.*, (1) the elimination of the noxious, putrefying and gas forming organisms and (2) the furthering of the appropriate ripening and an acceleration thereof. The first of the above aims is reached by the utilization of organisms that produce a high degree of acidity, whereas it is necessary for the attainment of the second aim that the organisms utilized possess the faculty of producing the appropriate products in the process of proteolysis. I will repeat here that the application of inoculation according to my method joins with the hygienic production and treatment of the milk in yielding a product practically free from undesirable microorganisms.

Similar considerations may be made with reference to the preparation of silage. Silage that has undergone the lactic acid fermentation is by far the best, both from the standpoint of cattle feeding as also from the standpoint of subsequent cheese manufacture. Every other silage is undesirable because, in spite of its satisfactory appearance and apparently low germ content, it is a carrier of butyric acid organisms.

The production of a lactic silage is best accomplished by following the accompanying general rules: (1) The use of siloes with impervious foundations; (2) a semi dried condition of the fodder;

(3) air exclusion from the mass obtained by the use of deep layers of silage and of strong packing whereby the temperature of the silage is kept between 35°C. and 40°C.; and (4) inoculation with lactic acid bacteria to best insure the desired results, especially when fodders are used which are not fit for a spontaneous lactic fermentation.

In conclusion it pleases me to be able to emphasize that the majority of my results have been verified in recent years by various authors, *i.e.*, Barthel, Boekhout, and De Vries, Burri, Esten, Evans, Hardings, Harrison, Hoffman, Löhns, Orla Jensen, and others.

## A METHOD FOR THE CULTIVATION OF ANAEROBES<sup>1</sup>

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In the course of a study of the bacteria causing spoilage in canned foods it became necessary to place large numbers of cultures under anaerobic conditions, and to devise anaerobic methods which would care for them with a minimum of expense of time and material.

A review of the literature showed that the most commonly recommended methods, which would be suitable for these purposes, were absorption of oxygen by alkaline pyrogallate and the replacement of air by an inert gas, usually hydrogen, or a combination of the two.

The chief objections to these methods are that they are difficult to apply, requiring complicated apparatus, are expensive and time consuming. We have encountered still another difficulty in that even the most carefully made containers are likely to leak air. In case of a leak, the pyrogallate solution is soon exhausted, or the hydrogen passes out very rapidly and is replaced by air. We have found that even the all-glass Noxy jar would not maintain anaerobic conditions for twenty-four hours in many cases. These jars are also of rather limited capacity and very expensive. The number of methods suggested would lead one to doubt the complete suitability of any one for all occasions, and experience with their use merely establishes the correctness of this impression.

Several years ago Dr. Wolbach suggested the method of Sellards which he had used successfully in his work on anaerobes.

<sup>1</sup> Contribution Number 36 from the Bacteriological Laboratories of the Kansas Agricultural Experiment Station.

This method makes use of metallic phosphorus for the removal of oxygen. Most authors mention this method of obtaining anaerobic conditions in their review of the literature, but so far as we have been able to find, none of them except Wolbach have advocated it. In the introduction to his article describing this method, Sellards (1904) makes the statement that up to that time no one had recommended the use of phosphorus for anaerobic work, although phosphorus is one of the most powerful absorbing agents of oxygen. Among the recommendations for its use, this author mentions that phosphorus is very convenient, that it requires no previous preparation, that it keeps well, and that its absorbing efficiency is very easily tested.

He mentions two possible difficulties; first, that the oxides of phosphorus formed during the absorption of oxygen might change the reaction of the medium employed; second, that the vapors of the elementary phosphorus might injure the nutrient media. He mentions not having had any difficulty from these sources.

The phosphorus method has been used in this laboratory for the past three years and we wish to recommend it for the cultivation of anaerobic bacteria. We have been able to culture all obligate anaerobes, which we have tried upon the surface of plates and slant agar. Obligate anaerobes also grow well in liquid media under anaerobic conditions produced by phosphorus. We have had no difficulty concerning the injury to the media. There is a slight rise in temperature within the jar as the phosphorus burns; this, however, exerts no detrimental effect upon the cultures. One great advantage of this method over others is that the power of phosphorus to absorb oxygen is so great that a rather small amount will absorb the oxygen within the jar, and enough will remain to absorb whatever may leak in during the incubation period. Sellards recommended the presence of an excess of alkali to absorb the phosphorus acid anhydrides. We have found that water serves the same purpose almost equally well. There is some theoretical objection to burning the phosphorus in the jar, in that elementary phosphorus is volatilized. We have had no difficulty from this source as far as the growth of bacteria is concerned, but some of the material is deposited



upon the surface of the plates and upon the cotton plugs, thus making the culture dishes unpleasant to handle. This has been overcome to some extent by covering the plates and cotton plugs with paper. Another source of trouble was encountered in labeling the tubes with a wax pencil. The phosphorus seemed to soften the wax of the ordinary red wax pencil, causing the labels to become blurred. Later a blue wax pencil was found which was not influenced in this way. We have also resorted to the use of paper labels held in place by rubber bands, since the excessive moisture in the jar will soon cause gummed labels to fall off.

Some difficulty was experienced in obtaining an anaerobic jar large enough to hold all the plates or cultures which we wished to run at one time, and at the same time, one which could be easily handled and remain air tight under a reduced pressure of three to four inches of mercury for several days. Quart "Lightning Seal" fruit jars have been used for test tube work to some extent. In this case, however, the sides of the jar and tubes must be protected from the burning phosphorus. This may be accomplished by strips of thin asbestos board.

As a large anaerobic jar, we have used an aluminum pressure food cooker (figs. 1 and 2), manufactured by the Pressure Cooker Company, of Denver, Colorado. The pressure gauge on the top is removed and this opening plugged. Just before using, the edge of the cover is heavily smeared with a rubber cement, similar to that used on stopcocks. This jar will retain a vacuum of 4 to 5 inches of mercury for as long as two weeks in the incubator at 37°C. (we have not tested it for longer periods.) Water is placed in the bottom of the jar, cultures are placed on the wire rack (fig. 2), an evaporating dish containing the phosphorus is placed in the jar and covered with a bit of wire gauze having a small opening in the center. This is to prevent the burning phosphorus from spattering. When all is ready, one of the stopcocks in the cover is opened and the cover is held so that it may be replaced quickly. A hot needle is passed through the small hole in the wire gauze, and the phosphorus is ignited. The cover is quickly replaced and screwed down firmly. The pet cock is shut off after a few seconds, and a careful watch main-

tained for leaks. As the phosphorus burns there is considerable positive pressure due to the increase in temperature. The pet cock should always be left open until this is nearly neutralized, otherwise leaks may develop. If there are leaks around the lid, they can be detected by the puffing out of phosphorus fumes. The burning usually ceases in a very few seconds, due to the exhaustion of oxygen; and the jar is filled with fumes. In glass jars these are seen to subside in about fifteen minutes if there is plenty of fresh water in the bottom. Anaerobic conditions are



FIG. 1. ANAEROBIC JAR WITH  
LID IN PLACE



FIG. 2. ANAEROBIC JAR WITH LID REMOVED

thus obtained about the cultures almost instantly. The rate at which oxygen dissolved in the medium, will diffuse out will depend upon the medium. Litmus milk and broth containing methylene blue are decolorized within a few hours. Deep agar tubes containing methylene blue are not decolorized to the bottom for about three days. Apparently oxygen diffuses out about as slowly as it diffuses into the media, since oxygen will diffuse into agar at the rate of about 1 cm. per diem. This is indicated by the fact that most vigorously growing anaerobes will grow to within one or 1.5 cm. of the surface of a deep agar tube.

The jar which we have described is of light weight, quickly sealed and very convenient. It may also be used with hydrogen or illuminating gas. If alkaline pyrogallate is used it must be

placed in a separate dish and not upon the bottom of the jar since these chemicals attack the metal. The capacity of the size which was used is about twenty-five plates or eighty test tubes of 15 mm. diameter. Everything considered, this is the most convenient and effective method of culturing anaerobes which we have yet encountered. One precaution which must be taken on all occasions, is not to allow bits of phosphorus to remain exposed to the air. These may not burn for several hours if moist, but as soon as they become dry they will burn and may set fire to the laboratory. The phosphorus in stock should be placed in firmly corked glass bottles and kept in the ice box. In this laboratory we have had some difficulty because the temperature became so high during the summer that the sticks melted together into a mass in the bottom of the container, thus making it difficult to remove. This trouble may be overcome by keeping the material in the ice box. Sellards recommends drawing out the phosphorus into small sticks. This is not at all necessary, and involves considerable trouble.

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## INFLUENCE OF VACUUM UPON GROWTH OF SOME AEROBIC SPORE-BEARING BACTERIA<sup>1</sup>

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In the course of a study of the bacteria present in canned foods, we found that certain aerobic spore-bearing bacteria were present in jars which showed no evidence of spoilage, if they were properly sealed. These results were reported from this laboratory in 1918 (Bushnell 1918), but no attempt was made to determine the types present.

Vaillard, in 1900 and 1902 examined bacteriologically, many cans of meat, and found living organisms in seventy or eighty percent of them. He believed that the bacteria survived in a dormant state in the cans from five to seven years. Among the spore-bearing types he isolated *B. subtilis* and *B. mesentericus*, (three varieties, *vulgatus*, *ruber*, and *fuscus*).

Deichsetter, in 1901, reported on the examination of preserved food provided for the Bavarian Army during a period of five years and failed to find microorganisms in canned foods, save in cans in which the food was sent in under suspicion. He considered that Vaillard's findings were probably due to faulty technic.

Pfuhl, in 1904, examined canned meats from five firms and found bacteria in 29 out of 106 cans. He considered that the findings of both Vaillard and Deichsetter were correct and that the difference in results was due to a difference in the care with which the foods were sterilized.

Very little work had been reported upon this point until 1919 when Weinzirl published the results of his findings on commercial

<sup>1</sup> Contribution Number 37 from the Bacteriological Laboratory of the Kansas Agricultural Experiment Station.

canned foods. He states that in commercial canned foods giving no evidence of spoilage, microorganisms were found in 179 out of a total of 782 cans, or in 23 per cent of the cans. The spore-bearers were practically the only organisms present, due to their superior resistance to the sterilizing process. Viable spores were found in 19.2 per cent of the non-leaking cans. Of the types of bacteria isolated, *B. mesentericus* predominated, with *B. subtilis* next. This author concludes that the living spores in commercial canned foods are unable to grow, due to the absence of oxygen, and that the vacuum is essential to the preservation of canned foods under the present method of processing.

Cheyney, in 1919, reported *B. mesentericus* in apparently perfect cans, which were given a standard processing.

Hunter and Thom, in 1919, made an examination of 530 cans of canned salmon and found 237 unsterilized. 234 of these cans contained the same organism of the *B. mesentericus* group, either in pure culture or in connection with other species. Only 13 showed active spoilage.

From the above it is evident that the aerobic spore-bearing types predominating in unspoilied canned foods belong to the *B. mesentericus* and *B. subtilis* groups of bacteria.

We may consider three reasons why these organisms predominate in foods under such conditions:

1. Spores of certain types predominate on the product as it goes into the container.
2. Spores of certain types are more resistant to heat than spores of other types.
3. The spores of certain types are not all destroyed during the processing period and those remaining are able to grow under conditions as they exist in the container.

From the results obtained in this laboratory, it is evident that *B. mesentericus* predominates, with *B. subtilis* second in number among the aerobic spore-bearing types. We have no idea of the number of each type upon the raw product as it went into the jars, so that it is not possible to consider this point except in so far as we may apply the work of Bruett (1919) upon the death of bacteria. She concluded that the death rate followed

the laws of monomolecular reactions. According to this law, if all spores were of equal resistance to heat, those present in the largest number would be the last to disappear. However, spores of different species of bacteria are not of equal resistance to heat, and while those of a particular species may follow this law, it cannot be applied in a comparison of the thermal death rate of different species.

We have found that the several cultures of *B. mesentericus* with which we have worked, are less resistant than the strains of *B. subtilis*. Regardless of this decreased resistance *B. mesentericus* predominated among the organisms isolated. Our cultures had grown for some time upon culture media and the resistance may have changed by this treatment. Weinzirl considers that *B. mesentericus* predominates in canned foods because of its superior heat-resisting qualities.

Lawrence and Ford (1916) state that the spores of *B. subtilis* survive steaming one and one-fourth hours in the Arnold sterilizer and autoclaving up to and including 19 pounds pressure but are usually destroyed at 20 pounds. The *B. mesentericus* spores survived one hour in the Arnold sterilizer and autoclaving at 19 pounds pressure, being killed by 20 pounds pressure. These statements would indicate that their cultures of *B. subtilis* were somewhat more resistant than those of *B. mesentericus*.

From some previous work upon these two types we had considered that *B. mesentericus* could grow in the absence of oxygen more readily than *B. subtilis*. Cheyney in 1919, also calls attention to this fact in his recent article on the bacteriology of canned foods.

We have occasionally isolated *B. mesentericus* from the deeper layers of agar in our search for anaerobes, but we have never isolated *B. subtilis* under such conditions, although it has been found on the surface several times. It must be admitted that we did not make quantitative determinations of the types present in each jar. The predominance merely means that in the routine isolation of colonies more of the *B. mesentericus* type were isolated, although we are convinced that this organism did predominate in the jars.

In an attempt to determine why *B. mesentericus* predominated, we undertook some experiments, using these two types. The types used were isolated from jars of asparagus and were rapid spore formers, although they had been grown in the laboratory for more than a year.

The thermal death point of the spores of the *B. subtilis* culture used in these experiments was from ninety to one hundred and twenty minutes in steam at 98°C.; for *B. mesentericus* eighty to one hundred minutes. The time at which all are killed depends to some extent upon the numbers present.

For the experimental work, the organisms were grown upon plain extract agar from four to six days. The growth was scraped from the medium and suspended in a small amount of sterile normal saline. This was shaken in a heavy walled bottle with glass beads and filtered through sterile cotton to remove clumps. The suspension was heated at 80°C. for twenty minutes to kill the vegetative cells.

*Experiment 1.* In this experiment we wished to determine the influence of different amounts of air upon the growth of the organisms. The number of spores indicated in the table were added to tubes of extract broth, 0.50 per cent N/1 acid to phenolphthalein pH 5.9. In this case the column of air above the medium was about 5 cc. The tubes were exhausted to various points and sealed. The results are shown in table 1.

From this table we may conclude that there was slight growth of both organisms during fifty-one days incubation at room temperature in the less exhausted tubes.

*Experiment II* was conducted in order to determine the influence of varying amounts of salt and air upon the growth of *B. subtilis* and *B. mesentericus*. In this case, known amounts of air were left above the liquid.

An attempt was made to remove all possible traces of air from the medium. To do this, the tubes were partially filled with a known amount of broth to which varying amounts of salt had been added. The tubes were then drawn out to a slender neck, as close to the liquid as possible, and heated in a seamer for fifteen minutes. The tubes were next cooled in cold water, and



1 cc. of a suspension of spores added. The spores were suspended in salt solutions to correspond to that in the tube, so that the salt concentration was not changed. All the tubes were filled to a mark on the constriction, with broth containing corresponding amounts of salt. A certain per cent of this was then removed and the tubes exhausted and sealed at the mark. The tubes were then incubated at 37°C. for twenty-seven days and plates made.

TABLE I

DAYS INCUBATION	B. SUBTILIS					B. MESENTERICUS				
	Num- ber spores added	Tubes exhausted to following mm. Hg. on manometer				Num- ber spores added	Tubes exhausted to following mm. Hg. on manometer			
		175	350	525	685		175	350	525	685
5	3110	2740	2220	3000	2280	1300	1830	1730	1370	1440
5	31	25	18	43	58	13	32	31	36	30
12	3110	1170	7000	1900	1000	1300	2100	2000	1090	640
12	31	69	60	16	71	13	31	18	19	36
19	3110	2300	3500	2500	8600	1300	3200	1830	1020	120
19	31	50	41	50	30	13	60	38	13	20
41	3110	3200	4000	5000	6000	1300	4000	1990	1260	260
41	31	49	29	11	12	13	90	72	27	8
51	3110	9000	7000	3200	5000	1300	4000	2460	1120	370
51	31	90	120	12	11	13	150	71	18	11

Since each tube had a slightly different amount of broth added, it was necessary to calculate how many organisms were present in each centimeter of liquid in the beginning. These numbers are in one column and the number at the end of the incubation period in a parallel column. The volume of air varied from 0.3 cc., in the tubes containing 1 per cent air to 1.5 cc. in the tubes containing 25 per cent air, each tube being of somewhat different volume from the others. The results are shown in tables 2 and 3.

These tables show the same as table 1, that there is some growth in these tubes. There is an interesting point relative to the action of increased amounts of salt. In every case, when the average is taken for all tubes in the same concentration of salt, there is an increase over that of a lower concentration. This point is somewhat more in evidence in connection with

TABLE 2

*Influence of varying amounts of salt and air upon the growth of B. subtilis in broth*

EXHAUSTED TO MM. Hg.	AIR	1 PER CENT NaCl		2 PER CENT NaCl		4 PER CENT NaCl	
		Bacteria added per cubic centi- meter	Bacteria determined per cubic centimeter	Bacteria added per cubic centi- meter	Bacteria determined per cubic centimeter	Bacteria added per cubic centi- meter	Bacteria determined per cubic centimeter
	<i>per cent</i>						
0	5	167,000	100,000	149,000	190,000	175,000	320,000
	10	146,000	150,000	157,000	200,000	165,000	250,000
	25	152,000	117,000	151,000	240,000	151,000	210,000
50	5	146,000	210,000	151,000	102,000	157,000	320,000
	10	152,000	160,000	157,000	110,000	165,000	240,000
	25	141,000	180,000	149,000	110,000	162,000	270,000
100	5	149,000	111,000	151,000	160,000	162,000	180,000
	10	162,000	220,000	143,000	240,000	143,000	270,000
	25	162,000	220,000	146,000	260,000	149,000	
Average .....		153,000	163,000	150,000	179,000	158,000	258,000
175	1	113,000	129,000	98,000	28,000	104,000	164,000
	5	135,000	76,000	119,000	27,000	124,000	384,000
	10	135,000	112,000			117,000	104,000
	25	113,000	102,000	129,000	158,000	113,000	424,000
350	1	113,000	89,000	129,000	200,000	124,000	448,000
	5	123,000	78,000			117,000	342,000
	10	132,000	98,000	124,000	200,000	118,000	476,000
	25	123,000	55,000	104,000	318,000	107,000	688,000
525	1	113,000	37,000	114,000	152,000	108,000	444,000
	5	113,000	84,000	114,000	256,000	113,000	436,000
	10	118,000	126,000	129,000	240,000	104,000	428,000
	25	123,000	76,000	114,000	220,000	118,000	416,000
685	1	123,000	97,000	124,000	26,000	119,000	516,000
	5			119,000	44,000	113,000	458,000
	10	141,000	48,000	119,000	100,000	113,000	454,000
	25	135,000	145,000	132,000	160,000	114,000	472,000
Average .....		123,000	90,100	118,000	153,000	114,000	416,000

INFLUENCE OF VACUUM UPON GROWTH OF BACTERIA 289

TABLE 3

*Influence of varying amounts of salt and air upon growth of B. mesentericus in broth*

EXHAUSTED TO MM. Hg.	AIR	1 PER CENT NaCl		2 PER CENT NaCl		4 PER CENT NaCl	
		Bacteria added per cubic centi- meter	Bacteria determined per cubic centimeter	Bacteria added per cubic centi- meter	Bacteria determined per cubic centimeter	Bacteria added per cubic centi- meter	Bacteria determined per cubic centimeter
	<i>per cent</i>						
0	5	340,000	320,000	328,000	280,000	323,000	720,000
	10	307,000	220,000	314,000	310,000	323,000	750,000
	25	345,000	450,000	328,000	260,000	340,000	860,000
50	5	328,000	340,000	328,000	450,000	341,000	940,000
	10	340,000	260,000	328,000	440,000	341,000	870,000
	25	323,000	290,000	345,000	480,000	328,000	Broken
100	5	323,000	Broken	314,000	440,000	328,000	560,000
	10	328,000	210,000	292,000	290,000	314,000	630,000
	25	340,000	190,000	345,000	360,000	353,000	810,000
Average .....		331,000	285,000	324,000	367,700	332,300	767,500
175	1			15,300	35,000	17,400	23,000
	5	15,300	29,000	15,500	36,000	15,300	25,000
	10	14,900	24,000	17,700	36,000	15,300	45,000
	25	17,400	22,000	14,300	60,000	14,500	24,000
350	1	15,200	18,000			17,700	22,200
	5	16,000	11,000	15,900	26,000	17,400	24,000
	10	17,700	32,000	18,500	15,000	15,900	48,000
	25	18,100	31,000	15,700	34,000	17,700	29,000
525	1	16,600	26,000	15,300	27,000		
	5	17,600	26,000	14,900	32,000	17,400	24,000
	10	18,600	18,000	16,000	27,000	15,700	34,000
	25	16,000	27,000	16,200	22,000	16,600	29,000
685	1			17,400	29,000	16,000	25,000
	5	17,700	15,000	16,000	27,000	15,700	30,000
	10	16,000	13,000	18,600	30,000	17,400	24,000
	25	16,100	12,000	15,700	20,000	17,400	21,000
Average .....		16,600	21,500	16,900	30,400	16,700	31,300

*B. mesentericus* than with *B. subtilis*. The very small amount of air remaining in these tubes apparently has no influence upon the amount of growth. There were relatively no more organisms present in tubes merely sealed and with 25 per cent volume of air, than there were in those tubes which were exhausted and with but 1 per cent of the volume of air. We believe that the organisms in the tube exhausted to 685 mm. were under as completely anaerobic conditions as it is possible to obtain.

According to Bitting and Bitting (1916) an ordinary tin can shows a vacuum of about four inches of mercury when exhausted at a temperature of 130°F. and tested at 85°F.

We may conclude from the results obtained, that the degree of vacuum plays no part in the destruction of the spores of these two organisms. When one per cent of salt is present, there may be a slight decrease in the twenty-seven days of incubation. This is somewhat more marked in higher vacuum than in the lower, but the differences are not marked. In the presence of larger amounts of salt, there appears to be an actual increase in the number of viable bacteria. This is apparently not due to accidental conditions, since we have made numerous parallel determinations and find that the averages of those determined at the end of the incubation period are two or three times as high as the number added. Why there should be a decrease in the presence of 1 per cent, and an increase in the presence of 4 per cent salt we are unable to say. Of course there is considerable variation in the determinations, but the averages indicate a real increase.

*Experiment III.* This experiment was set up parallel with that of experiment II, except that varying amounts of acid were added to the broth. The organisms were treated in the same way as those in the last experiment, except that they were suspended in acid broth after heating to kill the vegetative forms. The tubes were incubated at 37°C. for twenty-three days for *B. subtilis* and twenty-two days for *B. mesentericus*. The results are shown in tables 4 and 5.

Apparently *B. subtilis* spores are more sensitive to acid than those of *B. mesentericus*. The degrees of vacuum had no influence

TABLE 4

*Influence of varying amounts of acetic acid and air upon the growth of B. subtilis in broth*

EXHAUSTED TO MM. Hg.	AIR	PERCENTS OF N/1 ACID AND pH							
		0.5 per cent pH 5.6		1 per cent pH 5.60		2 per cent pH 4.30		4 per cent pH 4.00	
		Added	Deter- mined	Added	Deter- mined	Added	Deter- mined	Added	Deter- mined
	<i>per cent</i>								
0	5	715,000	429,000	800,000	173,000	890,000	241,000	800,000	184,000
	10	783,000	450,000	858,000	219,000	872,000	205,000	890,000	170,000
	25	716,000	457,000	737,000	265,000	715,000	256,000	828,000	145,000
50	5	813,000	392,000	907,000	223,000	800,000	234,000	858,000	176,000
	10	872,000	465,000	737,000	232,000	828,000	247,000	858,000	166,000
	25	783,000	472,000	813,000	175,000	761,000		860,000	186,000
100	5	858,000	408,000	872,000	209,000	858,000	198,000	813,000	52,000
	10	813,000	430,000	858,000	250,000	847,000	236,000	761,000	100,000
	25	783,000	472,000	813,000	273,000	828,000	234,000	800,000	130,000
Average . . . . .		802,000	443,000	836,000	224,000	822,000	231,000	823,000	146,000
175	5	409,000	256,000	410,000	214,000	410,000	157,000	391,000	
	10	383,000	250,000	451,000	270,000	409,000	171,000	391,000	70,000
	25	409,000	303,000	383,000	190,000	451,000	192,000	400,000	170,000
350	5	400,000	212,000	400,000	250,000	409,000		409,000	140,000
	10	374,000	270,000	429,000	230,000	440,000	148,800	409,000	103,000
	25	451,000	303,000	440,000	137,000	409,000	141,600	400,000	210,000
525	5	440,000	266,400	366,000	128,000	409,000	162,400	451,000	100,000
	10	383,000	259,400	429,000	178,000	463,000	146,400	391,000	180,000
	25	410,000	273,600	409,000	210,000	366,000	165,600	409,000	110,000
685	5	463,000	276,000	440,000	230,000	410,000	176,800	400,000	140,000
	10	400,000	258,000	391,000	220,000	488,000	162,400	429,000	190,000
	25	391,000	216,000	463,000	225,000	429,000	158,400	400,000	131,000
Average . . . . .		409,400	262,300	418,000	206,900	424,400	162,100	407,700	140,000

upon the decrease in numbers, but the higher amounts of acid were more active than the smaller amounts. As with *B. subtilis* in the presence of salt, there is a marked decrease even in the tubes with smaller amounts, but unlike the higher amounts of

TABLE 5

*Influence of varying amounts of acid and air upon the growth of B. mesentericus in broth*

EXHAUSTED MM. Hg.	AIR	PER CENTS OF N/1 ACID AND pH							
		0.5 per cent pH 5.60		1 per cent pH 5.00		2 per cent pH 4.30		4 per cent pH 4.00	
		Added	Deter- mined	Added	Deter- mined.	Added	Deter- mined	Added	Deter- mined
	<i>per cent</i>								
0	5	116,000	90,000	114,000	120,000	114,000	240,000	109,000	120,000
	10	104,000	80,000	114,000	150,000	102,000	220,000	102,000	130,000
	25	102,000	89,000	102,000	140,000	118,000	280,000	101,000	230,000
50	5	108,000	54,000	108,000	140,000	102,000	240,000	106,000	140,000
	10	111,000	56,000	96,000	68,000	118,000		91,000	150,000
	25	104,000	75,000	104,000	160,000	108,000		111,000	150,000
100	5	111,000	45,000	114,000	110,000	91,000	120,000	96,000	113,000
	10	101,000	52,000	109,000	110,000	109,000	190,000	102,000	113,000
	25	109,000	48,000	104,000	200,000	102,000		108,000	140,000
Average .....		107,000	66,000	107,000	199,000	107,000	215,000	103,000	151,000
175	5	40,000	19,200	42,900	16,800	47,600	18,000	38,300	49,600
	10	41,000	16,000	33,200	22,800	45,100	20,000	37,400	33,600
	25	47,600	19,200	36,000	23,200	39,000	36,000	42,900	47,200
350	5	42,900	14,800	36,600	25,200	42,900	49,000	46,300	36,800
	10	44,000	15,200	45,100	26,800	40,900	35,000	46,300	48,000
	25	40,000	12,000	41,000	19,200	46,300	60,000	45,100	65,600
525	5	40,000	14,400	40,900	14,000	36,100	28,000	41,000	43,300
	10	41,000	16,500	44,000	16,800	48,800	30,000	38,300	59,200
	25	39,000	16,000	39,100	14,000	47,600	27,000	37,400	40,000
685	5	44,000	16,000	41,000	18,400	45,100	17,000	39,100	48,000
	10	48,800	18,800	40,000	21,600	45,100		46,300	43,000
	25	45,000	12,800	40,000	13,600	45,100	34,000	44,000	51,200
Average .....		42,800	15,980	40,300	19,360	44,100	31,700	41,800	47,140

salt there is a marked decrease in the higher concentrations of acid. Even sealing seems to cause a marked decrease in the number of viable spores of both types. Perhaps if the incubation period had been lengthened there would have been a still greater

decrease in case of *B. subtilis*. Parallel tubes which were not sealed showed heavy growth of these organisms in all concentrations of salt, but only a trace of growth in the higher amounts of acid. In the sealed tubes there was a very faint visible trace of surface growth in the tubes containing larger amounts of air. This was easily broken up and did not re-form on standing. The plates made from these tubes checked much better than would generally be expected, when it is considered that organisms of this type produce such heavy surface growths in open tubes. However, these organisms do not form such adherent growths in sealed tubes, and by vigorous shaking a fairly uniform suspension may be obtained.

In the case of *B. mesentericus* there was much less marked action of acid. Either there is not so much decrease in the higher amounts of acid or there is a slight increase of this organism after the initial decrease. At the end of the incubation period there were about the same numbers as at the beginning of the experiment.

In *Experiment IV* an attempt was made to determine the influence of the amount of air and salt upon the thermal death point of *B. subtilis* and *B. mesentericus*. The results are shown in tables 6 and 7. The tubes and spore suspensions were prepared as above.

It is evident from these tables that *B. mesentericus* spores are somewhat more easily killed by heat than those of *B. subtilis*. We have found this to be true in numerous other tests upon the thermal death point of these organisms.

The amount of salt or the amount of air has practically no influence upon the thermal death point, particularly in the case of *B. subtilis*, the larger amounts of salt seeming, however, to protect the organisms to some extent.

*Experiment V* shows the influence of acetic acid and varying amounts of air upon the thermal death point of *B. subtilis* and *B. mesentericus*.

In this experiment the spores were prepared as above described. The liquid in which the spores were suspended during the heating was extract broth plus 0.5 percent; 1 per cent; 2 per cent; 4 per

cent of N/1 glacial acetic acid, giving the pH as shown in the tables.

Two centimeters of this acid broth were added to the tubes which were then heated in the steamer for fifteen minutes and cooled as rapidly as possible to remove air from the liquid. To

TABLE 6

*Influence of varying amounts of salt and air upon thermal death point of B. subtilis spores. Original numbers of spores added 32,000,000 per cubic centimeter*

SALT <i>per cent</i>	MM. Hg.	TIME OF HEATING AT 98°C.			
		15 minutes	30 minutes	60 minutes	120 minutes
1	Open	460,000	140,000	6,100	15
	175	110,400	40,000	7,600	31
	350	520,000	170,000	6,500	49
	525	640,000	200,000	8,300	15
	685	400,000	121,000	9,300	32
	Average .....		426,000	134,000	7,560
2	Open	670,000	180,000	8,700	38
	175	500,000	250,000	9,800	21
	350	400,000	120,000	6,400	42
	525	580,000	210,000	14,600	71
	685	700,000	160,000	10,400	24
	Average .....		570,000	184,000	9,980
4	Open	860,000	480,000	18,000	36
	175	840,000	200,000	14,500	56
	350	840,000	190,000	33,600	66
	525	473,000	200,000	26,000	82
	685	860,000		44,000	72
	Average .....		774,600	302,500	27,200
General average .....		590,200	206,800	14,910	44

these tubes was added 1 cc. of a heavy suspension of spores suspended in acid broth, similar to that in the tubes. The tubes were then filled to the mark with similar broth and the volume noted. Amounts equal to 5 per cent, 10 per cent, 25 per cent of the total volume were removed from the tubes. They were



then exhausted to the points desired and sealed at the mark. The tubes were all placed in a steam sterilizer and heated for one hour. Tables 8 and 9 show the results obtained. Since each tube was of slightly different volume the liquid remaining after removing the above amounts would contain slightly different

TABLE 7

*Influence of varying amounts of salt and air upon the thermal death point of B. mesentericus spores. Original number of spores added 3,780,000*

SALT	MM. Hg.	TIME OF HEATING AT 95°C.				
		15 minutes	30 minutes	60 minutes	120 minutes	
per cent	1	Open	8,000	700	17	2
		175	2,400	200	8	0
		350	1,000	130	34	0
		525	3,600	500	17	0
		685	1,600	200	8	0
		Average .....		3,320	346	17
2	}	Open	2,300	1,100	98	0
		175	1,100	800	30	0
		350	1,400	400	27	0
		525	1,000	520	39	0
		685	1,000	700	74	0
		Average .....		1,360	704	53
4	}	Open	1,700	390	69	0
		175	1,000	640	56	0
		350	2,300	700	88	3
		525	1,400	320	20	0
		685	1,500	200	70	0
		Average .....		1,580	450	61
General average .....			2,087	500	44	1

numbers per cc. The reduction in numbers in this experiment is so striking that the numbers are not included in the tables in each case. The average number for each cc. of the liquid remaining in the tubes was about 37,400,000 per centimeter for *B. subtilis* and 1,760,000 for *B. mesentericus*.

The above tables show a very marked influence of acid upon the thermal death point of both organisms. The fact that canned fruits and vegetables containing, or treated with acid, kept so much better than fruits and vegetables containing no acid, was

TABLE 8

*Influence of varying amounts of acetic acid and air upon the thermal death point of B. subtilis spores*

MM. Hg.	AIR	PERCENTS OF N/1 ACETIC ACID			
		0.5 per cent pH 5.5	1 per cent pH 5.10	2 per cent pH 4.5	4 per cent pH 4.10
175	per cent				
	5	590	9	3	0
	10	490	0	0	0
	25	700	0	2	0
Average.....		593	3	1	0
350	5	482	0	0	0
	10	142	0	2	0
	25	336	0	6	0
	Average.....		320	0	2
525	5	43	0	0	0
	10	113	0	0	0
	25	46	0	0	0
	Average.....		67	0	0
685	5	44	0	2	1
	10	600	3	4	2
	25	702	1	0	0
	Average.....		448	1	2
Open.....		372	0	3	1
General average.....		360	1	2	1

formerly thought to be due to the fact that the acid inhibited growth. From the results which we have obtained, we are inclined to believe that the keeping is due, not so much to the influence of the acid in inhibiting growth as to the fact that most or all of the organisms present are killed by the heating process.

The few remaining are probably unable to grow to any extent in the highly acid medium.

Here, also, the amount of air present in the containers has no influence whatever upon the thermal death point of the bacteria present.

TABLE 9

*Influence of varying amounts of acetic acid and air upon the thermal death point of B. mesentericus spores*

MM Hg.	AIR  per cent	PERCENTS OF N/1 ACETIC ACID			
		0.5 per cent pH 5.0	1 per cent pH 5.10	2 per cent pH 5.50	4 per cent pH 4.10
175	5	4	1	0	0
	10	10	1	0	0
	25	8	0	0	0
Average .....		7	1	0	0
350	5	11	0	0	0
	10	2	1	0	0
	25	16	1	0	0
Average .....		9	1	0	0
525	5	13	2	0	0
	10	10	1	0	0
	25	10	3	0	0
Average .....		11	2	0	0
685	5	2	2	0	0
	10	5	0	0	0
	25	16	2	0	0
Average .....		7	1	0	0
Open .....		12	2	0	0
General average .....		9	1	0	0

*Experiment VI* shows the influence upon the thermal death point of *B. subtilis* and *B. mesentericus* of several of the more common organic acids found in fruits. The spores were prepared as described in experiment V and placed in the acid solutions

after heating at 80°C. for twenty minutes to kill the vegetative forms. The results are shown in table 10.

TABLE 10

*Influence of organic acids upon the thermal death point of B. subtilis and B. mesentericus spores*

	TIME OF HEATING	PERCENT OF N/1 ACID ADDED TO BROTH			
		0.5 per cent	1 per cent	2 per cent	4 per cent
B. subtilis added 7,305,000 per cubic centimeter					
Lactic	<i>minutes</i>				
	15	74,400	17,300	3,200	273
	30	3,400	2,000	50	0
	60	80	10	0	0
B. mesentericus added 2,500,000 per cubic centimeter					
Lactic	15	18,000	100	90	10
	30	8,200	0	0	0
	60	13	0	0	0
B. subtilis added 17,500,000 per cubic centimeter					
Tartaric	15	520,000	410,000	52,000	2,000
	30	73,000	54,000	3	3
	60	600	576	2	0
B. mesentericus added 2,145,000 per cubic centimeter					
Tartaric	15	14,000	1,630	4	10
	30	1,200	220	0	0
	60	39	3	0	0
B. subtilis added 27,000,000 per cubic centimeter					
Citric	15	2,000,000	110,000	200	500
	30	240,000	600		200
	60	1,200	4	4	0
B. mesentericus added 17,000,000 per cubic centimeter					
Citric	15	24,000	60	50	12
	30	2,150	48	0	0
	60	0	0	0	0

The results show that there is very little difference in the action of these organic acids. The acetic is perhaps a little more effec-

tive, but not enough to be of practical importance. This table also shows the influence of the amount of acid, and also the fact that *B. mesentericus* is somewhat more easily destroyed by heat than *B. subtilis*.

#### SUMMARY

From the results obtained, we are inclined to believe that *B. mesentericus* predominates in canned foods because it is capable of growing to some extent in absence of air, rather than because its spores are more heat resistant than some other types of aerobic bacteria.

The amount of vacuum under which spores of these organisms are placed during the heating does not influence the thermal death point.

The small amount of acid present had but slight retarding influence upon the growth of these organisms in air, but did have a marked influence upon the thermal death point. It may be that the beneficial influence of acid upon the keeping of canned foods is due more to the lowering of the thermal death point than to the inhibition of growth of the organisms.

The amount of air remaining above the liquid has little influence upon the growth of these bacteria, since sealing the tubes prevents all but minimum growth. This inhibiting influence is more marked in case of *B. subtilis* than in case of *B. mesentericus*.

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# SUBSTITUTION OF BROM-THYMOL-BLUE FOR LITMUS IN ROUTINE LABORATORY WORK

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One of the common methods for the qualitative determination of acid or alkali production by bacteria is to inoculate nutrient extract broth containing various carbohydrates with litmus as an indicator.

Litmus possesses the disadvantage of being reduced by many organisms to a colorless compound, thus rendering it useless as an indicator. Clark and Lubs (1917) mention that the sulphon-phthalin indicators are much more resistant to bacterial action than indicators like methyl red or litmus. They suggest, because of certain preliminary tests, that indicators like brom-thymol-blue and brom-cresol-purple might be used to advantage in replacing other indicators which are now used in making indicator media.

With this suggestion in mind, an experiment was undertaken to find a method of preparing media to determine qualitatively acid or alkali production by bacteria, which would be easy to prepare, and more sensitive than litmus; and one in which the reaction could be quickly determined at any time during the incubation period.

In this experiment, sugar free broth was used, which was prepared as follows:

One pound of ground lean meat was digested for two hours with 1 liter of distilled water. After cooking, the broth was filtered through absorbent cotton into a flask and sterilized in the autoclave at 18 pounds pressure for twenty minutes. When cold the broth was inoculated with a culture of *Bact. saccharolyte* (Rivas)

and incubated at 37°C. for forty-eight hours to render the medium sugar-free. Then the medium was sterilized in the Arnold for twenty minutes; 10 grams of peptone and 5 grams of sodium chloride were added; the reaction was adjusted to pH 7.0 with brom-thymol-blue; the medium was again steamed for twenty minutes, the reaction readjusted, and the medium filtered.

To determine the amount of brom-thymol-blue which would inhibit acid production by microorganisms, fifty cubic centimeters of sugar free broth were placed in each of fifteen flasks.

TABLE 1

*The influence of brom-thymol-blue upon acid production by Bact. coli-communis. Using 1 per cent glucose broth, initial pH 7.0, increasing amounts of a 0.2 per cent alcoholic solution of brom-thymol-blue inoculated with 0.1 cc. of an eighteen hour broth culture*

LENGTH OF INCUBATION	DILUTIONS OF BROM-THYMOL-BLUE														
	0	1:125,000	1:62,500	1:41,666	1:31,250	1:25,000	1:20,750	1:17,810	1:15,610	1:13,900	1:12,500	1:11,360	1:10,420	1:9,650	1:8,875
<i>hours</i>															
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2½	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
3	++	++	++	+	+	+	+	+	+	+	+	+	+	+	+
4	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

- = No acid production; + = moderate acid production; ++ = strong acid production.

To each flask was added sufficient 0.2 per cent alcoholic solution of brom-thymol-blue to give the concentrations as shown in the tables. Equal amounts of the media were placed in five test tubes of similar size, and the tubed media were sterilized in the autoclave at 18 pounds pressure for twenty minutes.

When the tubes were cold, one cubic centimeter of a sterile 20 per cent glucose solution was added, under aseptic conditions, to each tube. The tubes were incubated at 37°C. for forty-eight hours, in order to make certain that none of them became contaminated during the process of adding the glucose solution.





which were much higher than need to be used in actual acid determinations.

*Bact. paratyphosum A*, *Bact. dysenteriae*, *Bact. sanguinarium*, *Bact. pullorum*, and *Bact. enteritidis* were also tested out in the same dilutions shown in the tables. Results were obtained closely approximating those for *Bact. typhosum*.

It has been found by experience that a 1:41,666 solution of brom-thymol-blue gives the most desirable concentration for colorimetric comparison. The data in the tables show that this concentration can be used in the media without inhibiting the

TABLE 4

The influence of brom-thymol-blue upon acid production by *Bacillus subtilis*, using media and dilutions as in table 1

LENGTH OF INCUBATION	DILUTIONS OF BROM-THYMOL-BLUE														
	0	1:125,000	1:62,500	1:41,666	1:31,250	1:25,000	1:20,750	1:17,810	1:15,610	1:13,900	1:12,500	1:11,360	1:10,420	1:9,650	1:8,875
hours															
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
5	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
6	++	++	++	+	+	+	+	+	+	+	+	+	+	-	-
7	+++	+++	+++	+++	+	+	+	+	+	+	+	+	+	+	+

production of acid. This dilution is easily made by adding 12 cc. of a 0.2 per cent alcoholic solution of the indicator to every liter of sugar free broth before it is put into the fermentation tubes.

Media prepared in this way are now used in this laboratory by the students, and have been found successful for all their qualitative acid production tests.

It has also been used by a member of the department in determining acid production by a certain bacterium, upon about twenty carbohydrates. In this particular instance, readings of the reaction of the cultures were made every day over a period of four weeks of incubation.

The advantages of this medium are:

1. Brom-thymol-blue includes the neutral point in its range of hydrogen-ion concentration, so that the medium can be adjusted to exact neutrality before being inoculated.

2. A medium containing sufficient brom-thymol-blue to act as an indicator will not inhibit acid production.

3. Brom-thymol-blue is not reduced by microbial action.

4. The reaction of a carbohydrate medium containing brom-thymol-blue can be recorded at any time during incubation.

5. Changes in color with slight changes in hydrogen-ion concentration are more marked with brom-thymol-blue than with litmus.

6. Brom-thymol-blue is easier to prepare than litmus.

7. Heat does not affect brom-thymol-blue during sterilization.

8. The reaction of carbohydrate broth containing brom-thymol-blue can be read by artificial light, but this is impossible with litmus.



## THE USE OF DOMESTIC METHYLENE BLUE IN STAINING MILK BY THE BREED METHOD

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Some difficulty has been experienced in staining preparations of milk by the Breed method. This is well summarized in the Standard Methods for the Bacteriological Examination of Milk,<sup>1</sup> as follows: "Some methylene blue now on the market in powder form is very unsatisfactory in that solutions will dissolve the milk films, or will wash them with an even blue color in which the bacteria fail to show distinctly. Old or unfiltered stains are to be avoided as they may contain troublesome precipitates."

During the past summer an effort was made at this laboratory to find means to correct these difficulties. To this end five samples of methylene blue, all reported to dissolve the milk films, were obtained from H. J. Conn, Chairman of the Committee on Technique of the Society of American Bacteriologists. All proved unsatisfactory when the stain was made up in a saturated aqueous solution using distilled water, and irregular results were obtained upon making up the stain with tap water. It was found that the addition of a small amount (approximately a quarter gram per liter) of  $\text{CaCO}_3$  in the form of precipitated chalk caused one of the samples to stain without dissolving the films, and the difficulty with another sample was corrected after the  $\text{CaCO}_3$  had stood in the solution for 48 hours. On account of the poor solubility of the  $\text{CaCO}_3$ ,  $\text{Na}_2\text{CO}_3$  was tried with uniformly good results.  $\text{NaHCO}_3$  seems to work equally well. This indicates that the addition of small amounts of  $\text{Na}_2\text{CO}_3$  to aqueous solutions of those methylene blues which are worthless because they

<sup>1</sup>Third edition, 1921, p. 15, Amer. Pub. Health Assoc., Boston.

dissolve the milk preparations, may render them satisfactory for use.

Methylene blue for bacterial stains comes on the market both as a zinc double salt and as a zinc-free hydrochloride. Methylene blue "for bacilli" is generally the zinc salt but may contain various quantities of the free hydrochloride. The pure hydrochloride is a dark greenish-blue crystal and is handled under the trade names of "Medicinal" or "U. S. P. " A solution of the pure hydrochloride usually gives a dark blue stain to dried films of milk which becomes deeper the longer the preparation is left in the solution. The deep blue of the casein can be taken out by decolorizing with alcohol. The bacteria do not lose their stain as rapidly as the casein, thus making it possible to prepare a good preparation with this form of methylene blue. Methylene blue "for bacilli," which may be the pure zinc salt but may contain certain amounts of the free hydrochloride, may be any color from a greyish-red to a reddish-blue. An aqueous solution of the zinc salt will stain satisfactorily; but it is insoluble in alcohol and cannot be used in the Loeffler formula. Usually decolorization with alcohol is unnecessary with zinc salt solutions. An aqueous solution of the zinc salt which contains very little or none of the pure hydrochloride may possibly cause the washed out appearance mentioned in the Committee report.

Aqueous staining solutions frequently become grossly contaminated with bacteria and molds, so much so that organisms from this source may be found on the smears stained in these solutions. To avoid this difficulty Loeffler's alkaline methylene blue has been tried with uniformly good results. Later it was found that any solution of methylene blue prepared in 30 per cent alcohol was as satisfactory as the Loeffler's formula, and also prevented the growth of organisms in the staining solution. These results indicate that stains prepared in alcoholic solutions are to be preferred to the aqueous solutions recommended in the Standard Methods Report; but on account of the insolubility of the zinc salt in alcohol, either the medicinal methylene blue or some methylene blue "for bacilli" which is not largely made up of the zinc salt must be used.

# JOURNAL OF BACTERIOLOGY

## Contents

### JANUARY, 1922—VOL. VII—No. 1

- HILDA HEMPL HELLER. Certain Genera of the Clostridiaceae. Studies in Pathogenic Anaerobes. V.  
O. ISHII. Studies upon Agglutination in the Colon-Typhoid Group of Bacilli.  
O. ISHII. A Study of Spontaneous Agglutination in the Colon-Typhoid Group of Bacilli.  
ALBERT C. HUNTER. The Sources and Characteristics of the Bacteria in Decomposing Salmon.  
S. A. KOSER AND W. W. SKINNER. Viability of the Colon-Typhoid Group in Carbonated Water and Carbonated Beverages.  
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An Investigation of American Stains.

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J. HOWARD BROWN. Hydrogen Ions, Titration and the Buffer Index of Bacteriological Media.  
J. E. RUSH AND G. A. PALMER. On Decreasing the Exposure Necessary for the Gelatin Determination.  
HAROLD MACY. Chart of the Families and Genera of the Bacteria.

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MAY, 1922

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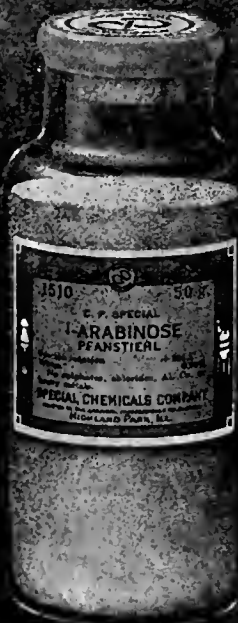
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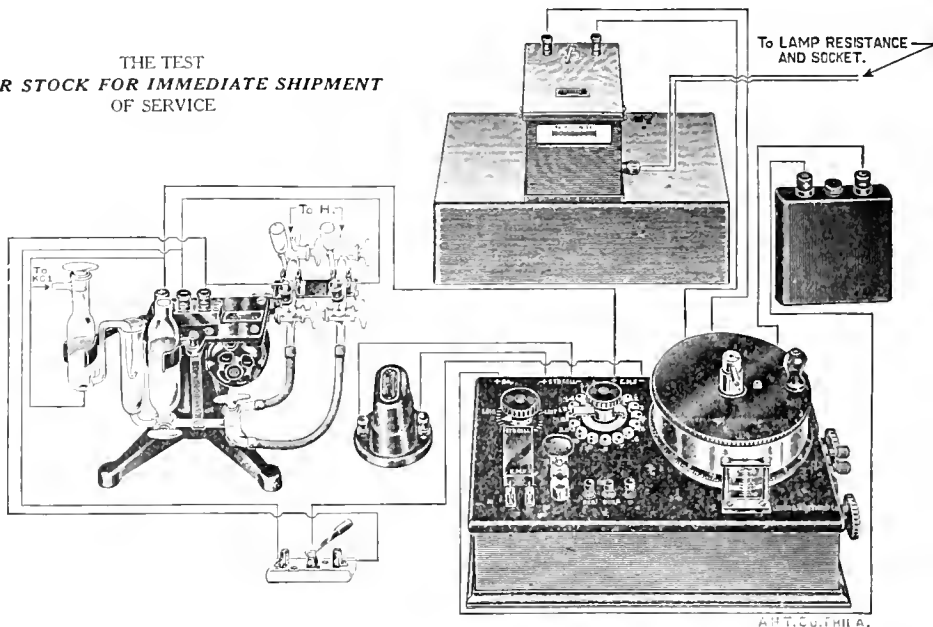
## CONTENTS

J. HOWARD MUELLER. Studies on Cultural Requirements of Bacteria. I	309
J. HOWARD MUELLER. Studies on Cultural Requirements of Bacteria. II	325
SELMAN A. WAKSMAN. A Method for Counting the Number of Fungi in the Soil	339
LETHE E. MORRISON AND FRED W. TANNER. Studies on Thermophilic Bacteria. I. Aerobic Thermophilic Bacteria from Water	313
ROBERT G. GREEN. An Apparatus for the Rapid Measurement of Surface Tension	367

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# STUDIES ON CULTURAL REQUIREMENTS OF BACTERIA. I

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Received for publication November 1, 1921

## INTRODUCTION

With the exception of a few such media as Ushinsky's and Frankel's, which are of interest only from the fact that they demonstrate the ability of certain species of bacteria to synthesize protein and other complex physiological substances from simple salts, all the pathogenic bacteria are cultivated for practical purposes on empirical mixtures containing infusions of meat, the digestion products of protein and so forth. Most pathogens either fail to grow, or produce very scant growth on simple synthetic media. Many fail to multiply even on meat extract-peptone media, but flourish after the addition of serum, blood, and similar materials. It occasionally happens that a lot of stock media, prepared with all due regard to known components and hydrogen ion concentration will fail to grow such organisms as pneumococci, although other lots prepared in the same way have been successful.

Numerous attempts have been made to amplify the use of synthetic media by the addition of known compounds as a source of nitrogen or of some particular chemical grouping which has been suspected of playing a part, but as far as progress in the direction of routine cultivation of bacteria on media of known composition is concerned, the results have been uniformly disappointing. On the other hand, there seems to have been very little effort made to attack the problem from the angle of an analysis of the basic factors supplied by the physiological mixtures which are

known to induce growth. One is struck by the lack of reference to the identity of the components of meat extract or meat infusion which seem to be so universally favorable. Purine bases are mentioned, but apparently on insufficient experimental grounds. In fact the only piece of work which the writer has found in which an effort was made to determine this point was reported by Armand-Delille (1913), who claims to have substituted arginine for meat infusion in media used for the cultivation of the tubercle bacillus. Other work (Long, 1919) indicates that this organism is able to grow on a great variety of substances, and one is not justified in applying the conclusions of Armand-Delille to other types of bacteria without further experimental evidence. The possibility of obtaining some light on the requirements of pathogenic bacteria from this point of view is, however, fairly promising, and it is the purpose of the present paper to outline the method of approach and to cite a number of preliminary experiments which serve to indicate the lines along which work may be done. The results already obtained with two of the factors which it has been possible to single out and follow up has been made the subject of two preliminary reports (Mueller, 1920), and will shortly appear in greater detail.

A word regarding the purpose of such an investigation and the results which can be expected from it may not be out of place. The present empirical methods of media preparation are generally admitted to be uncertain from the standpoint of result, and wasteful in materials used. It may be justly questioned, however, whether a more thorough understanding of requirements will lead to a radical change in our methods of preparation of media. For example, even if certain amino acids and organic bases should prove to be the only essential factors beside salts and perhaps carbohydrates, the difficulty and expense of obtaining them in pure form might well prevent their extensive practical use, and it is quite possible that peptone and meat will continue to be their most available source. It should, however, be possible to understand the reasons for the uncertainty of results and occasional failures now existing, and perhaps to guard effectively against them, even though with experienced workers, they occur rather rarely.

Perhaps the most important results to which success in such a piece of work might lead, are the applications of the findings to problems of more general biological importance, particularly to those of animal metabolism. For, whatever may prove to be the nature of these substances which cause growth of bacteria, they are largely or entirely components of animal tissue, and it is probable that they are either needed also by the animal body and supplied by plant or other sources, or else are synthesized by the animal itself to fill some metabolic requirement. When it is possible to catalogue the substances required by pathogenic bacteria for growth, it will probably be found that most of them are either required by, or important in, animal metabolism, and while many of them will surely be compounds at present familiar to the physiological chemist, it is equally probable that some will be new, or at least of hitherto unrecognized importance. This point is sufficiently clear in the light of many recent publications in connection with the relation of vitamins to the growth of bacteria and of yeast.

Probably of no less importance will be the results from the standpoint of the classification of bacteria. Doryland (1920) has discussed this question at some length, and it is quite possible that unexpected relationships or dissimilarities in bacterial species may develop on the basis of food requirements. That related species among pathogenic bacteria do have similar needs cannot be questioned. The colon-typhoid group grow easily on simple meat extract broth. The streptococci and pneumococci require the presence of an infusion of meat, the meningococci and gonococci usually grow poorly without the additional presence of "hormones," blood serum, etc., while the influenza bacillus needs a substance associated with hemoglobin. It is quite possible that the more fastidious types of bacteria may require some of the factors necessary for the more easily growing forms, plus one or more additional substances. For example, if growth of the typhoid bacillus depends on the presence of three compounds in meat extract broth, A, B, and C, then the pneumococcus will perhaps fail to grow unless A, B, and C are supplied together with D and E, and so on to such organisms as the

gonococcus and others. Such a conception will at least serve as a working hypothesis upon which to begin investigation.

The choice of organisms with which to work must be governed by several considerations. In the first place, since several factors are probably involved in the growth of all parasitic bacteria, it would be well to select a species having a somewhat limited number of requirements. In this way one might hope to single out one or two factors at a time for identification and, using these as a basis, proceed to the study of others. On the other hand, bacteria with too simple requirements should be avoided, because of the probability that such organisms possess the power of using a variety of different materials having no immediate relationship to the components of meat extract and peptone. The colon bacillus, for example, can grow on Uschinsky's or other simple media, about as well as on ordinary extract broth, and for that reason would not be suitable for the purpose at hand. A further point to be considered is that since the work will probably extend over a long period of time, the type of organism selected must be such that its cultural requirements will not change materially during preservation on culture media.

For these reasons, the pneumococcus-streptococcus group was selected as being probably the most satisfactory. These organisms grow well on meat infusion broth, but poorly or not at all on extract broth. While blood or serum improves the growth, neither is essential. In addition, these organisms can be preserved almost indefinitely and kept at what should be quite uniform food requirements. The method by which this is attempted will be described fully below.

The original plan of the work was to start with meat infusion-peptone broth, to eliminate such factors in its composition as could be managed experimentally, and to substitute known compounds, such as amino-acids, purine bases, etc., or failing in this, to determine, if possible, the chemical nature of the material removed from the media. As will appear, the work has been little more than begun, since the difficulties in substituting known compounds for the meat infusion have proved unexpectedly great.



## METHODS

*Preparation of cultures*

Three types of pneumococci, I, II and IIA, together with a strain of *Streptococcus hemolyticus*, were used. The pneumococcus strains were each passed through two mice, and the streptococcus through one mouse and stock cultures were made from the heart blood of the second mouse into small tubes containing about 1 cc. of sterile human blood. The latter was obtained in the usual way from the median basilic vein and transferred from a syringe to small sterile tubes, each tube containing two or three glass beads. The tubes were then shaken until defibrination was complete, and incubated twenty-four hours to insure sterility before being inoculated from the heart blood of the mice. After inoculation the tubes were incubated eight to ten hours until smears showed that the organisms were multiplying, and they were then stored in the ice box. For transplants, ordinary meat infusion peptone broth containing 0.1 per cent glucose and brought to pH 7.4 to 7.8, were used. A small loopfull of the blood culture was transferred to the broth, and after incubating eighteen to twenty-four hours, the culture was used, in the case of the pneumococci for the inoculation of a second similar tube of meat infusion broth. These two cultures are called respectively the A and B cultures. Experimental media were inoculated from the B cultures in the case of pneumococci, and from the A tubes with streptococci. In this way it is believed that the food requirements of the bacteria remain reasonably constant. The stock culture remains alive for long periods of time, up to six months or more. After being opened repeatedly, old tubes gradually dry up, and such cultures have been transferred to fresh blood tubes, using the same technic, and always passing the strain through one mouse to guard against a gradual change in cultural requirements in spite of the blood media. It is not unlikely that significant quantities of blood may be carried over into the test media with only a single passage intervening, but it certainly is not enough to produce growth on unsuitable media, although it cannot be overlooked in estimating the value of results obtained.

*Determination of limiting hydrogen-ion concentration for strains employed*

It was found, by inoculating a series of plain broth media with pH ranging from 6.8 to 8.6, that the pneumococci found conditions suitable for growth between pH 7.4 and 8.0, while the hemolytic streptococcus grew well throughout the same range, and even as far down as pH 6.8. For the adjustment of experimental media, therefore, a reaction of pH 7.4 to 7.8 has been used for all the strains of test organisms. In cases where several experimental lots of media are prepared at one time, the adjustment of the reaction is facilitated by the direct addition of phenol red to the entire lot. It is a simple matter to reach the particular pH desired by using a solution of phenol red of 0.02 per cent concentration, in the proportion of 1 cc. to 25 cc. of media. Normal or semi-normal NaOH (moderately free from carbonate) is then added drop by drop until the color is distinctly red, but not purple. One can quickly learn to recognize the correct color without the need of standard color tubes. In the case of unusually dark colored media the reaction can be adjusted approximately by this means and finished by the usual method of dilution and addition of more indicator followed by comparison with standard tubes. Experiment showed that several times this amount of phenol red could be added to media without any influence on the growth of the organisms in question.

*Limiting range of osmotic pressure*

Although moderate variations in osmotic pressure are probably of little importance in culture media, an experiment was carried out in broth made up with varying concentrations of NaCl. The test organisms grew equally well in salt-free broth and through several intermediate concentrations up to an equivalent of 2 per cent NaCl, so that with ordinary care the question of osmotic pressure apparently need not enter into consideration.

*Presence of glucose*

In preliminary work with the pneumococci it was observed that occasionally a lot of meat infusion was met with which

failed to give growth, although prepared with all due attention to hydrogen ion concentration, etc. The addition of as little as 0.025 per cent glucose to such media at once improved them to such a degree that marked growth occurred, and with twice this quantity, growth was approximately as heavy as in the average broth prepared without glucose. While the point has not been verified experimentally, it is not improbable that most meat contains small quantities of glucose or other carbohydrate, and that occasionally this may be much diminished or absent. For routine purposes, therefore, and in all experimental media, 0.1 per cent glucose is added. This quantity is insufficient to produce acid in amounts great enough to kill the cultures in twenty-four hours, or to interfere with agglutination with specific sera.

#### *Inorganic constituents*

Little is known definitely of the salt requirements of bacteria. There is some evidence in the literature to indicate that they are moderately elastic, and in any case such minute traces as may be required, must occur in the peptone and meat infusion used as routine media. Where these or similar substances are omitted, small quantities of inorganic salts may well be required. The nature of these can only be determined when purified organic compounds can replace the meat infusion and peptone.

To guard as far as possible against failure of experimental media to support growth through lack of inorganic material, the same salt mixture which is used in Usebinsky's and other similar media has been employed instead of simple NaCl, in the preparation of all experimental media in which the ordinary meat infusion is not used.

To simplify the preparation of media, the salts are all dissolved in twice the concentration required, together with glucose, and phenol red is added to this solution. Media are prepared by adding an equal volume of meat infusion or other solution to this preparation, thereby reducing the concentrations of the constituents to that desired. The composition of the solution is shown below, and it will be referred to subsequently as "glucose-salt solution."

	<i>per cent</i>
NaCl.....	1.0
MgSO <sub>4</sub> .....	0.04
CaCl <sub>2</sub> .....	0.02
HK <sub>2</sub> PO <sub>4</sub> .....	0.2
Glucose.....	0.2
Phenol red.....	50 cc. of 0.02 per cent solution per liter

If all the components except the potassium phosphate are dissolved, and the solution diluted almost to the final volume before this substance is added, calcium phosphate is not precipitated, although a good deal is probably lost in the precipitate which usually forms on adjusting the reaction and boiling.

#### *Sterilization of media*

Since it has been shown that ten minutes autoclaving at 10 pounds pressure is even less destructive to sugars than the Arnold temperature for three quarters of an hour, this method has been used in most of the work. Practically no change in pH occurs, and contaminations are exceedingly rare.

#### *Recording of results*

When growth has continued for twenty-four hours, the degree of turbidity is recorded by comparing the tube with a set of seven tubes containing suspended BaSO<sub>4</sub> (Koser and Rettger, '19) ranging from the faintest trace of a cloud to a suspension as heavy as the best growth obtained with these organisms on good media. Negative growth is shown by "O," and any degree of growth by a number corresponding to the series number of the BaSO<sub>4</sub> standards. This affords a means of correlating experiments done at different times, although of course it is not quantitative. It usually happens, however, with the pneumococcus cultures, that after optimum growth has been reached, autolysis follows very quickly. Within a few hours, a tube which has shown growth equal to standard tube nos. 4 and 5 will clear up and become simply opalescent. With Type I this happens quite regularly in less than forty-eight hours on good media, since maximum growth is reached by this strain in fifteen to eighteen hours. Types II and IIA grow more slowly, but are often autolyzed in forty-eight

hours. In recording growth, such tubes are marked "A." This autolysis is of course not a criterion of good growth, for in experimental media, scanty growth is also followed by clearing up. The streptococcus appears not to show this phenomenon.

*Preliminary experiments on infusion broth*

The growth of the test organisms on peptone-free infusion and on peptone water alone was first investigated.

Lot 1.	{	Meat infusion.....	25	cc.
		Glucose-salt solution.....	25	cc.
		Peptone <sup>1</sup> .....	0.5	gm.
Lot 2.	{	Meat infusion.....	25	cc.
		Glucose-salt solution.....	25	cc.
Lot 3.	{	Peptone.....	0.5	gm.
		Water.....	25	cc.
		Glucose-salt solution.....	25	cc.

LOT NUMBER	pH	TYPE I	TYPE II	TYPE IIA	STREPTOCOCCI
1	7.8	A	A	4	5
2	7.6	A	5	2	6
3	7.8	4	0	0	0

It appears from this experiment that peptone-free infusion is practically as satisfactory a medium for the growth of the pneumococci and streptococci as the usual meat infusion broth containing 1 per cent of peptone. On the other hand, with the exception of the strain of Type I pneumococcus, peptone water alone will not support growth. As a working hypothesis, it has been assumed that these organisms have a nitrogen requirement supplied by peptones or amino acids, and also a need for certain accessory substances supplied by meat infusion. Therefore, on the basis of such a supposition, meat infusion seems to contain not only the accessory substances, but also peptones, amino acids or other sources of available nitrogen. The problem

<sup>1</sup> Throughout the work, "Difco" Peptone has been used for the sake of uniformity.

is complicated by this fact, since it is necessary to separate by some means the accessory substances from the nitrogen supply before either one can be studied separately.

*The need for an accessory factor*

Before describing the various methods by which a separation of the accessory factors of meat infusion was attempted, an experiment will be described which strengthens the probability that such substances, other than protein degradation products, are necessary. It is possible that only amino acids or peptones might be required for growth, but that in the preparation of commercial peptone some essential amino acid, as, for example, tryptophane, is wholly, or for the most part, altered or destroyed. It is quite conceivable that unstable groupings other than tryptophane may be present in the original protein molecule, which may withstand moderate heating in neutral solution and thus be present in meat infusion among the amino acids or peptones in a soluble form, and yet be almost or quite lacking in commercial peptone. If such were the case, a whole protein hydrolyzed by trypsin or erepsin, together with salts and glucose, would probably serve as a complete culture medium. A specimen of commercial casein after several day's digestion with trypsin was, as a matter of fact, found to be quite satisfactory without the presence of infusion, for the pneumococcus and streptococcus. However, when the casein was purified by three precipitations from  $\text{Na}_2\text{CO}_3$  solution by acetic acid, washed with alcohol and ether and then digested as before, growth was negative. The following protocol shows the results of such an experiment, in which casein was prepared directly from milk. The "erude" casein is the first precipitate obtained by acetic acid, the "pure" casein has been three times reprecipitated and finally washed in alcohol and ether.

The two preparations of casein were dissolved in 0.5 per cent  $\text{Na}_2\text{CO}_3$  and digested with a small quantity of trypsin (Fairchild) at  $37^\circ$  under toluol for two weeks. At the end of this time, the two solutions were boiled and filtered. Twelve cubic centi-

meters of each filtrate represented about 0.5 gm. casein. The following media were prepared:

Lot 1.	{	Digest of impure casein.....	12 cc.
		Water.....	13 cc.
		Glucose-salt solution.....	25 cc.
Lot 2.	{	Digest of pure casein.....	12 cc.
		Water.....	13 cc.
		Glucose-salt solution.....	25 cc.

LOT NUMBER	pH	I		II		III		STREPTOCOCCI	
		24 hours	48 hours	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours
		1	8.0	6	5	0	6	6	6
2	8.0	0	0	0	1	0	0	0	0

Control experiments showed that the digest of pure casein was not inhibitory, for the addition of meat infusion produced heavy growth.

The above experiment was confirmed several times, using different preparations of casein, always with the same result. The conclusion seems warranted that while crude casein contains some accessory substance, this is not a part of the protein molecule (and hence probably not an amino acid or polypeptide grouping), and can be easily removed by purification of the protein by standard means.

*Possibility of another source of growth accessory substance free from protein nitrogen*

By the use of the term "accessory substance" in connection with these studies, it is desired to avoid, as far as possible, the conception of vitamins. There is now abundant evidence in the literature that vitamins, particularly the water-soluble vitamin, may be as essential for certain microorganisms as for animals, but there is little probability that the accessory factor or factors of meat infusion is in any way connected with the water soluble vitamin. In the first place, muscle tissue is believed to be low in vitamin. In the second place, experi-

ments with the pneumococcus and streptococcus have shown that "protein-free milk," which does contain the water soluble vitamins, is almost without activity when substituted for meat infusion in media. To economize space, protocols of these experiments are omitted.

It is not impossible that physiological extracts other than meat infusion might supply the accessory factors in a form more free from other nitrogenous compounds such as amino-acids, than the latter, and in a few experiments it has been possible to show that some other preparations, notably one from blood, and one from spinach leaves, gave growth when mixed with peptone and only scant growth in its absence. The blood was diluted with water, acidified, boiled and filtered. The spinach leaves were dried, ground fine and extracted with water. In the case of the latter, initial extraction of the dried powder with ether did not remove the accessory substances, and boiling with repeated changes of alcohol for several hours extracted only a small part. It would perhaps be possible to develop a technic along either line for the preparation of a solution of the accessory factors sufficiently free from protein nitrogen to investigate the nature of the requirements of the test organisms by the addition of pure amino acids, but it has seemed more satisfactory first to exhaust as far as possible the more obvious methods for a separation of the meat infusion.

*Attempts to separate the growth accessory factors from the amino acids of meat infusion*

1. *Repeated extraction of meat.* While carrying out some experiments along another line, it was observed that the test organisms grew as well on a trypsin digest of the insoluble meat residue remaining after the preparation of meat infusion which had first been thoroughly boiled out in three changes of water, without the addition of any meat infusion, as upon the usual peptone broth. This suggested the possibility that the growth accessory material might be extracted from the coagulated protein with some difficulty, and might be partially separated in this way from amino acids, etc. Chopped beef was, therefore,



soaked in cold water in the proportion of 1 pound of meat to 500 cc. of water, and heated to 55° for a few moments and strained. The residue was boiled for five minutes with a second 500 cc. of water, and strained, and the extraction repeated a third time. The first extract was boiled to remove coagulable material, and all three extracts were filtered. Media were prepared in the following way, and after inoculation, gave the results indicated:

	EXTRACT I	EXTRACT II	EXTRACT III	PEPTONE	H <sub>2</sub> O	GLUCOSE SALT	GROWTH 36 HOURS			
							I	II	IIA	Streptococci
	cc.	cc.	cc.	grams	cc.	cc.				
1	25			1.0		25	5	7	7	6
2	25					25	1	1	2	5
3		25		1.0		25	3	3	4	4
4		25				25	0	1	1	3
5			25	1.0		25	5	3	5	6
6			25			25	0	0	0	0
7				1.0	25	25	3	0	1	0

Other experiments of the same kind have given like results, but there is enough irregularity so that the method is not ideal. It does, however, bring further evidence as to the presence of two classes of compounds in the meat infusion. Further work with the method, substituting known amino acids instead of peptone, may lead to more positive results.

2. *Chemical fractionation of meat infusion.* From the fact that commercial meat extract cannot replace meat infusion for the growth of the pneumococcus and streptococcus, one may suppose that heating, oxidation or simply long preservation may destroy the active material. If it can be shown experimentally that these or other comparatively simple chemical processes are destructive, plans for chemical separation can then be made in such a way as to avoid or minimize this difficulty. Accordingly, a few simple tests were made on the influence of such obvious procedures as suggested themselves. It was found that drying on the water bath, long boiling (two to three hours) in neutral or moderately acid solution (2.5 per cent HCl) and

exposure to mild oxidizing or reducing agents in no way diminished the power of infusion or destroyed its activity. It would seem that if hot alkali and strong acids are avoided, it should be possible to preclude loss of activity while carrying out simple precipitations on meat infusion.

This has been found to be the case, and several different reagents have been employed, without, however, any marked success in the separation of the constituents of the infusion. Precipitation with alcohol, up to 85 per cent concentration, carried out by preliminary evaporation of the infusion to a small, measured volume, followed by addition of the required amount of 95 per cent alcohol, has in several experiments yielded a precipitate which contains the growth accessory substances, since the test organisms grew on media prepared from the precipitate plus peptone, but not in the absence of peptone. The separation is somewhat more complete if the first precipitate is dissolved in a little water and reprecipitated. However, in either case, a part both of the growth accessory factors and of the amino acids remains in the alcohol filtrate, and it has not so far been possible to separate them quantitatively in this way.

Lead hydroxide, lead acetate, mercuric chloride and silver nitrate and baryta have all been tried, none of them with results satisfactory enough to follow up extensively. In general, the filtrates from these reagents will permit growth to some extent without the addition of peptone, and better growth in its presence, which is taken to indicate that the growth accessory fraction is not precipitated readily by these metals, while the amino acid fraction, perhaps in the form of peptone, is partially thrown down. By precipitation with tannic acid, followed by removal of the latter with  $\text{Ba}(\text{OH})_2 + \text{Pb}(\text{OH})_2$  in the usual way, very similar results are obtained.

While none of the methods of chemical separation have given successful results, they have at least shown that it is possible to submit meat infusion to such processes without loss of activity. It has, indeed, been observed that where a single preparation was put through several consecutive precipitations, the activity was gradually diminished or lost, but it is at least as possible that

this may have been due to the loss of some essential factor in a form which could not be recovered, as for example a tannin precipitate or an extremely insoluble silver combination, as to actual chemical decomposition of the substance. It is, in fact, quite probable that there are many essential factors present, and that progress can be made only as methods are developed which will enable one to single these out for identification. Such a method seems to have been found in the treatment of meat infusion with charcoal. By this means, one or more factors are removed from the infusion which may be again supplied by the addition of a small amount of peptone, or of a sulphuric acid hydrolysate of casein. In following up this lead, a considerable amount of work has been done, and a number of interesting observations made which have already been briefly reported (Mueller, 1920) and which will be dealt with in the next paper of this series.

#### SUMMARY

The purpose of this paper has been merely to outline the plan of work and describe the method followed in our studies upon the problem of the nutritional requirements of certain bacteria. The procedures as outlined in the section on methods will be used in such work unless modifications prove desirable. Sufficient evidence has been obtained from the experiments here reported to warrant belief that two classes of organic compounds (in addition to carbohydrates), are required for the growth of pneumococci and streptococci, the first supplied by protein degradation products, the second by extractives of meat. Both occur together in ordinary meat infusion, but they may be separated more or less completely in several ways. The necessity for a non-protein substance is shown most clearly by the failure of a trypsin digest of purified casein to support growth, while that of impure casein is satisfactory. The possibility of a separation of the two classes of compounds, as they occur together in meat is evident from experiments of several types, particularly by repeated extraction of meat, alcoholic precipitation of

meat infusion, and charcoal decolorization of heart infusion. There is every reason to believe that there may be several individual factors falling into each of these two groups.

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## STUDIES ON CULTURAL REQUIREMENTS OF BACTERIA. II

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In the introductory paper (Mueller, 1922) of this series of studies, it was intimated that by treating an infusion of beef heart with charcoal, two factors necessary in the growth of hemolytic streptococci were removed, and that these factors could be again supplied by the addition to the charcoal treated infusion of commercial peptone or of a sulphuric acid hydrolysate of casein. The main facts learned up to this time about these two growth determining substances have been already briefly reported (Mueller, 1920), and will be here presented in detail. Work upon these substances has not yet been completed in the sense of chemical isolation and identification, and it is hoped that after further investigation which is now under way, it will be possible to give a more definite report of their nature.

### TECHNIC

The general technic of preparation of media, adjustment of reaction, inoculation and recording of results, together with the method of carrying the test strain of streptococcus in culture, etc., has been fully described in the introductory paper (Mueller, 1922), and need not be repeated here.

*Removal of certain growth determining factors from beef heart infusion by charcoal, and reactivation of the charcoal treated infusion by means of peptone*

Three hundred cubic centimeters of a beef heart infusion, prepared by mixing chopped heart muscle and water in the pro-

portion of 1 pound meat to 500 cc. tap water, heating slowly to boiling, straining and filtering, was boiled for twenty-five minutes with 10 per cent "Norit," a commercial grade of wood charcoal used in sugar refining. The mixture was filtered through paper, and the colorless filtrate used in the preparation of the following media:

Lot 1	{ Decolorized infusion.....	25	cc.
	{ Glucose-Salt solution.....	25	cc.
Lot 2	Same plus peptone ("Difco").....	0.5	gm.
Lot 3	{ Original heart infusion.....	25	cc.
	{ Glucose-salt solution.....	25	cc.
Lot 4	Same plus peptone.....	0.5	gm.
Lot 5	{ Water.....	25	cc.
	{ Glucose-salt solution.....	25	cc.
	{ Peptone.....	0.5	gm.

Each lot was brought to pH of 7.4 to 7.8, filtered if necessary, tubed and sterilized at ten pounds steam pressure for ten minutes.

LOT NUMBER	GROWTH OF STREPTOCOCCUS IN TWENTY-FOUR HOURS
1	0
2	6 <sup>1</sup>
3	6
4	7
5	0

It is evident from this experiment, that beef heart infusion, prepared as described above, constitutes a perfectly satisfactory medium for the strain of streptococcus used, without the addition of peptone. Peptone water alone, moreover, even when supplied with glucose and an inorganic salt mixture will not support growth of the organism. When the infusion has been treated with Norit and filtered, it is no longer suitable for growth, but may be reactivated by the simple addition of peptone.

<sup>1</sup> Numbers refer to the standard turbidity scale composed of varying suspensions of BaSO<sub>4</sub>.

Many confirmatory experiments of the same nature have shown that within rather wide limits, the amount of charcoal used and the duration of boiling are without appreciable influence on the result, and as a standard method of producing the decolorized infusion, 2 per cent of charcoal and fifteen minutes boiling have been adopted.

The most obvious interpretation of the experiment is that the charcoal treatment removes certain substances, perhaps amino acids or polypeptides which occur also in commercial peptone, from the infusion. If polypeptides are concerned, they would probably be hydrolyzed by boiling with strong acid, and the resulting hydrolysate would not have the property of reactivating the decolorized infusion, while if one or more amino acids are responsible for the phenomenon, they would perhaps withstand acid hydrolysis.

*Reactivation of decolorized infusion with a sulphuric acid hydrolysate of casein*

A quantity of commercial casein was hydrolyzed by boiling for eighteen hours with a mixture of six times its weight of water and three times its weight of concentrated H<sub>2</sub>SO<sub>4</sub>. The resulting solution was freed from H<sub>2</sub>SO<sub>4</sub> by Ba (OH)<sub>2</sub>, the precipitate washed with water, and the filtrate and washings combined and concentrated. A quantity of the resulting hydrolysate equivalent to 0.5 gram of the original casein was used in the preparation of media as follows:

- Lot 1 { Decolorized infusion.....25 cc.  
Glucose-salt solution.....25 cc.
- Lot 2 Same plus casein hydrolysate..... 0.5 gm.
- Lot 3 { Water.....25 cc.  
Glucose-salt solution.....25 cc.  
Casein hydrolysate..... 0.5 gm.

LOT NUMBER	TWENTY-FOUR HOURS' GROWTH
1	0
2	7
3	0

It is apparent that the reactivating material will withstand fairly thorough acid hydrolysis, and is, therefore, probably, but not necessarily, not of a polypeptid nature, since it is known that protein hydrolyzed in this way is not completely reduced to the amino acid stage.

The further possibility exists that the reactivating material is not connected with the protein molecule at all, but is present as an impurity. If casein is reprecipitated several times from sodium carbonate solution, by means of acetic acid and the resulting product washed thoroughly by alcohol and ether, the "pure" casein so obtained will yield a hydrolysate which is just as active as that from crude commercial casein. It cannot, perhaps, be justly concluded from this experiment that the activating material is in fact a part of the protein molecule, for, as Funk (1920) has suggested, it may equally well be explained on the basis of a quantitative adsorption of the material from solution by the casein during precipitation. Since the material is known to be adsorbed quantitatively by charcoal, it is not impossible that it may also be taken up by other finely divided precipitates, although it is not adsorbed by such precipitates as  $\text{BaSO}_4$ , metallic sulphides, etc., as will appear later. For the present, we must await further evidence to show whether the substance is of protein or non-protein origin.

Since many of the experiments reported in the first paper of this series had shown that meat infusion was a rather difficult material to work with chemically, and since there are a number of fairly standardized methods for the partial separation of the amino acids in protein hydrolysates, the simpler experimental course seemed, at the time these preliminary observations were made, to attempt the separation and identification of the activating material from such protein hydrolysates. The possibility of re-extracting the material from the charcoal was considered, but since about 20 per cent of the total solids of the heart infusion are removed by charcoal under these conditions, it seemed probable that even though a method for re-extracting from the charcoal could be devised, the extract might contain a somewhat complex mixture of compounds difficult to characterize and work



with. It was, therefore, assumed as a working hypothesis, that one or more amino acids were involved in the reactivation and further efforts have been directed along the line of the isolation of such compounds.

*Preliminary observations on distribution of activating material*

If only a single substance were concerned in the reactivation, and it were one of the known amino acids, it should be possible to gain a clue as to its nature by using the hydrolysates of several different types of protein, and checking up a possible failure to reactivate in certain cases against a common deficiency, a method which has been widely used in work on animal metabolism. A number of proteins were, therefore, submitted to sulphuric acid hydrolysis, and tested with decolorized infusion. It appeared that the hydrolysates of casein, meat protein, edestin, egg white, and to a lesser extent, egg yolk and gelatine, were able to reactivate, while the material from wool, silk, and wheat gluten were inactive. No common deficiency was apparent, and the results in some cases were not always clear cut. Since it will shortly be shown that two substances are probably involved in the reactivation, it is quite possible that certain proteins may lack one and not the other, and it will be necessary to run through such a series of proteins again, testing for each substance individually, when the properties of the two have been more carefully investigated.

*Separation of an active fraction from casein hydrolysates with mercuric sulphate*

After trying a number of methods for the separation of an active fraction from hydrolyzed casein, with little success, it was finally found that a solution of mercuric sulphate in 5 per cent sulphuric acid would serve to throw down a precipitate containing most, if not all, of the activating material. This separation was first carried out upon a fraction containing the mono-amino acids of casein prepared by the butyl alcohol extraction method of Dakin (1918) which had been shown in preliminary experiments

to contain the greater part of the active material. It was found that the filtrate from the  $\text{HgSO}_4$  precipitate was no longer active, after precipitating the Hg with  $\text{H}_2\text{S}$ , and removing the  $\text{H}_2\text{SO}_4$  with  $\text{Ba}(\text{OH})_2$ , while the mercury precipitate, after freeing from Hg and  $\text{H}_2\text{SO}_4$  in the same way, was quite active.

Following this observation, preparations of the amino acids known to be precipitated by this reagent, namely, tryptophane, tyrosine, cystine and histidine, were obtained and their ability to reactivate the decolorized infusion either singly or in combination with each other was tested and found negative.

It was then found that mercuric sulphate would precipitate the active material directly from the casein hydrolysate, without resorting to the preliminary separation of the latter by means of butyl alcohol, using Dakin's method. Moreover, the sulphuric acid used in hydrolysis did not have to be removed with baryta, but could be neutralized by sodium hydroxide and the precipitation carried out in the resulting strong solution of sodium sulphate equally as well as in a solution free from salts. It is rather difficult to determine the optimal conditions of precipitation, but a considerable excess of  $\text{HgSO}_4$ , and not too high a concentration of  $\text{H}_2\text{SO}_4$  in the mixture seem to give the best results. As a standard procedure a weight of  $\text{HgSO}_4$  equal to that of protein taken, make up in 5 per cent  $\text{H}_2\text{SO}_4$ , and added to a hydrolysate which contains from 5 to 10 per cent amino acids and is nearly neutral in reaction, has been used. Precipitation is complete in about twenty-four hours. The filtrate, after freeing from Hg and  $\text{H}_2\text{SO}_4$ , may still show a slight ability to reactivate the decolorized infusion, but the precipitate is always strongly active; whether one of the two active materials to be described is removed more completely than the other by this method has not been definitely determined, but it is quite possible.

*Attempt to purify the active fraction by fractional precipitation with mercuric sulphate*

In the preparation of tryptophane by the method of Hopkins and Cole, advantage is taken of the fact that cystine is precipi-

tated more easily by that reagent, than tryptophane, in purifying the latter. It was, therefore, a logical procedure to attempt a separation of the active fraction of the mercuric sulphate precipitate by fractional precipitation with the same reagent. The results of a number of preliminary experiments in this direction gave evidence that the first crude mercuric sulphate precipitate contained two substances, both of which, together, were required for reactivation. One of these was easily reprecipitated by the addition of mercuric sulphate solution to the solution obtained from the first crude precipitate. The second was not completely reprecipitated even by the use of a considerable excess of the precipitant.

The following experiment will illustrate these facts. It will be observed that in place of an acid hydrolysate of casein, a commercial enzyme digest of milk proteins to the amino acid stage, called "aminoids" has been used. This was done for two reasons. In the first place, the process is less troublesome than the long continued acid hydrolysis, and in the second place the preparations obtained are somewhat more active than those from an acid hydrolysate. The latter, after a certain amount of chemical manipulation will often give rather weak growth in test media, corresponding to only a "2" or a "3" on the  $\text{BaSO}_4$  turbidity scale. It is realized that such a procedure is not beyond criticism, and that it perhaps strengthens the possibility that one is not dealing with amino acids, but with adsorbed non-protein material. However, it is equally possible that these physiologically active substances are partially destroyed or altered by long treatment with acid. At any rate, the main facts of each point established with aminoids have been checked with an acid hydrolysate of casein, and like results obtained.

Ten grams aminoids were dissolved in 100 cc. water and precipitated with 100 cc. of a 10 per cent solution of  $\text{HgSO}_4$  in 5 per cent  $\text{H}_2\text{SO}_4$ . After standing over night the bulky precipitate was filtered on a Buchner funnel, and washed with water. It was then suspended in water, made slightly alkaline with  $\text{Ba}(\text{OH})_2$  and decomposed with  $\text{H}_2\text{S}$ , allowing the reaction to take place for some time, with occasional warming. The precipi-

tated HgS was filtered off, and the heavily pigmented filtrate freed from Ba with  $H_2SO_4$ , and diluted to 100 cc. This crude preparation was tested for activity as follows:

Lot 1 { Decolorized infusion.....25 cc.  
 { Glucose-salt solution.....25 cc.  
 {  $HgSO_4$  ppt. fraction.....2.5 cc. (= 0.25 gm. casein)

Lot 2 Same plus  $HgSO_4$  ppt. fraction...0.25 cc. (= 0.025 gm. casein)

Lot 3 { Decolorized infusion.....25 cc.  
 { Glucose-salt solution.....25 cc.

LOT NUMBER	TWENTY-FOUR HOURS' GROWTH
1	4
2	3
3	0

To the remainder of the  $HgSO_4$  precipitate fraction was added 10 cc. of a 10 per cent solution of  $HgSO_4$ , and after standing over night, it was filtered. Both the precipitate and the filtrate were freed from Hg and from  $H_2SO_4$  as usual, and brought to a volume of 100 cc.

Lot 1 { Decolorized infusion.....25 cc.  
 { Glucose-salt solution.....25 cc.  
 { plus  
 {  $HgSO_4$  filtrate fraction.....1.0 cc.

Lot 2 Same plus filtrate fraction.....0.5 cc.

Lot 3 Same plus precipitate fraction.....1.0 cc.

Lot 4 Same plus precipitate fraction.....0.5 cc.

Lot 5 Same plus each fraction.....1.0 cc.

Lot 6 Same plus each fraction.....0.5 cc.

Lot 7 { Decolorized infusion.....25 cc.  
 { Glucose-salt solution.....25 cc.

LOT NUMBER	TWENTY-FOUR HOURS' GROWTH
1	1
2	0
3	0
4	0
5	5
6	5
7	0

This particular experiment has been selected from a number of a similar nature since it shows a fairly clean separation into two fractions. Not infrequently one fraction or the other will by itself reactivate the infusion to some extent and produce slight growth, but almost invariably both together are better.

Two points are illustrated in the above experiment. In the first place, an actual separation has taken place. It is not a distribution of the activating material through both fractions with a corresponding dilution, which might reduce the concentration below that required. In the second place, it is essential to have some idea about the quantity of material which is being added, best in terms of its equivalent of the original casein. Obviously, if, as is really the case, a quantity of casein hydrolysate, or of the first  $\text{HgSO}_4$  precipitate, equivalent to 0.25 gm. casein will reactivate 50 cc. of a mixture of decolorized infusion and glucose salt solution, one must not test a later preparation or fraction with a concentrated solution, using perhaps an equivalent of 5 grams of casein or more. If such were done, and the preparation found active, one might still have lost 95 per cent or more of the active material and not recognized it, for beyond a certain optimum, growth is not increased by multiplying the quantity of reactivating material considerably.

It has not been found possible so far to define the conditions of this precipitation so exactly as always to obtain a complete separation, and while it was useful in leading to the discovery that there were two substances involved, it has now been given up in place of a more certain method.

*Separation into two fractions by means of silver sulphate and baryta*

Because of the presence of considerable histidine in the crude  $\text{HgSO}_4$  precipitate, as shown by color reactions, a purification of the fraction by means of  $\text{Ag}_2\text{SO}_4$  and baryta suggested itself. As will be seen, this resulted in a simple method for the separation of the activating material into the two fractions. The method is not attended by the uncertainty of the  $\text{HgSO}_4$  fractional precipitation, and leads quite easily to a quantitative separation. The  $\text{Ag}_2\text{SO}_4$  precipitate, which contains the factor which will hereafter be referred to for the sake of brevity as "X," corresponds to the second  $\text{HgSO}_4$  precipitate, while the filtrate from the  $\text{Ag}_2\text{SO}_4$  fraction contains the "Y" as does also the filtrate from the second  $\text{HgSO}_4$  precipitation.

Aminoids are precipitated as described above with  $\text{HgSO}_4$ , and after freeing the precipitate from Hg and from  $\text{H}_2\text{S}$ , a hot saturated solution of  $\text{Ag}_2\text{SO}_4$  (or  $\text{AgNO}_3$  if the nitrate radicle will not interfere with further work) is added until an excess is present as shown by testing a drop with a drop of  $\text{Ba}(\text{OH})_2$ . Cold saturated  $\text{Ba}(\text{OH})_2$  solution is then added until precipitation is complete and the precipitate filtered or centrifuged off. The filtrate is freed from Ag with  $\text{H}_2\text{S}$  and Ba with  $\text{H}_2\text{SO}_4$ , and is concentrated to an equivalent of 10 per cent original aminoids over a low flame, and is found to contain the Y fraction, entirely free from X. The  $\text{Ag}_2\text{SO}_4$  precipitate contains the X fraction together with traces of Y, and it must be reprecipitated in the same way to free it entirely from Y. The Ag and Ba are removed as usual, the  $\text{H}_2\text{S}$  boiled out, the solution cooled and more  $\text{Ag}_2\text{SO}_4$  and  $\text{Ba}(\text{OH})_2$  added as in the first precipitation. This precipitate, when freed from Ag and Ba is found to contain the active X, quite free from Y. The test for active X or Y is of course made in the usual way, using decolorized infusion and glucose salt, together with a preparation known to contain Y or X as the case may be.

*Further purification of X fraction*

The  $\text{Ag}_2\text{SO}_4$  precipitate is always pigmented, and contains considerable histidine. It is needless to say that histidine, as

well as tryptophane, tyrosine and cystine have been tested out individually and collectively against known X and Y preparations and found to have no influence in producing growth under these conditions. The constancy of pigment in the X fraction, together with the fact of the disappearance of pigment from the heart infusion on boiling with charcoal, may be suggestive. However, the results obtained by the precipitation of this fraction with phosphotungstic acid, indicate that the pigment is not concerned in the action of the X fraction. By the addition of phosphotungstic acid to this fraction, in the presence of 5 per cent  $H_2SO_4$ , it was possible in several experiments to obtain a filtrate quite free from pigment, which when freed from phosphotungstic acid with  $Ba(OH)_2$  and the excess of Ba removed, contained an active X factor. Such a solution, evaporated on a watch glass yielded a semi-crystalline residue. The phosphotungstic precipitate also contains a small amount of the X, but in the single experiment in which it was attempted to learn quantitatively how it was distributed by using diminishing quantities against a constant amount of Y, the filtrate seemed to have about 75 per cent of all the X in the fraction. Unfortunately, these observations were made with a single solution of phosphotungstic acid, and all subsequent preparations of the reagent have destroyed the activity of the X fraction entirely. Up to the present, therefore, all that can be said of the X fraction is that it is apparently not in any way connected with the pigment nor with the histidine which it contains. It is hoped that further work will throw more light on this factor.

*Further purification of Y fraction*

The  $Ag_2SO_4$  filtrate, or Y fraction has proved to be somewhat simpler to work with than the X fraction. When evaporated, after freeing quantitatively from Ag and Ba, it is semi-crystalline. It contains a varying quantity of tyrosine and perhaps some tryptophane. By precipitation with a small quantity of  $HgSO_4$  and allowing the material to stand over night, any tryptophane is thrown out, together with part of the tyrosine. The resulting filtrate, after removing Hg and  $H_2SO_4$ , contains active Y.

It may be evaporated nearly to dryness, and after standing over night on ice to allow tyrosine to separate, may be filtered, the tyrosine washed out thoroughly with cold water, and the filtrate and washings, which still contain the active Y, evaporated further, with small additions of alcohol, to crystallization. The material which separates in the first crystallization is made up of microscopic round plates or spheres with no definite crystal form. After one or two recrystallizations these are seen to be made up of needles, and finally they crystallize out as shining, colorless, microscopic leaflets, often with obtuse angles and several sided, for the most part in rosettes; but when single crystals can be made out, they are hexagonal. These crystals are apparently a new amino acid, containing sulphur, and a detailed account of their composition and properties will appear shortly. Unfortunately, it cannot at this time be stated definitely that they constitute the Y substance. The earlier crops of indefinitely crystalline material are highly active, as are also the mother liquors. A quantity of solid weighing as little as 0.00001 gram has reactivated 25 cc. of a mixture of decolorized infusion and glucose salt solution in the presence of an active X preparation. However, on further recrystallization the activity is apparently lost, but the activity of the mother liquor also slowly disappears, and it is, therefore, not yet clear whether the sulphur containing crystals are some tautomeric form or oxidation product of the Y, or whether they are in no way related. In the meantime, a study of the properties of the sulphur compound will perhaps lead to methods by which it will be possible to answer this question.

#### DISCUSSION OF RESULTS

Since pathogenic bacteria such as the streptococci are biologically adapted to growth on animal tissues, it is more than probable that the chemical substances required by them for growth are constituents of the animal body and hence probably of importance in animal life. It is evident that studies on the cultural requirements of such bacteria may, therefore, lead to results equally as important to the student of animal nutrition as to



bacteriologists. In the case of the two substances which have been described in the present paper, one may anticipate that they may develop significance for animal metabolism as further information on their properties is gained. Whether they are related to the vitamins, and constitute, as Funk (1920) has suggested a Vitamin D, connected with deficiencies in certain proteins, is a question which can so far not receive an answer. The writer has preferred to avoid the conception of "vitamin" as far as possible in the experimental approach to the problem. In any case, the method as it has developed, offers a simple biological test for the presence of these compounds, which has the very great advantage that it may be quickly carried out. All the test solutions are easily prepared, and the growth test itself requires only twenty-four or at most forty-eight hours for completion. With this advantage, it should prove possible to isolate and identify these compounds unless their properties are such that decomposition or molecular rearrangement follows their purification. Work is being carried on with both fractions, and it is hoped to have more data available for report in the near future.

#### CONCLUSIONS

1. Peptone-free beef heart infusion plus glucose and inorganic salts constitutes a satisfactory medium for the hemolytic streptococcus.

2. Short boiling of heart infusion with 2 per cent wood charcoal ("Norit") removes some component of the meat infusion and renders it no longer suitable for the streptococcus.

3. Such an inactive infusion may be reactivated by the addition of small quantities of peptone or acid hydrolysate of certain proteins, such as casein and edestin.

4. Acid hydrolysates of such proteins, as wool, silk and wheat gluten are not suitable for reactivation.

5. The activating material may be precipitated from hydrolysates of casein by means of  $\text{HgSO}_4$ .

6. It may be separated into two fractions, active only when mixed together, by means of fractional precipitation of the first

HgSO<sub>4</sub> precipitate by HgSO<sub>4</sub>, or precipitation by Ag<sub>2</sub>SO<sub>4</sub> and baryta.

7. So far as has been learned, known amino acids will function in place of neither of these fractions.

8. The silver sulphate precipitate or X fraction does not depend for its activity on the pigment. It escapes precipitation by phosphotungstic acid under certain conditions, but is readily destroyed by this reagent.

9. The silver sulphate filtrate, or Y fraction contains a considerable quantity of a new sulphur containing amino acid, the relation of which to the active Y has not yet been demonstrated.

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# A METHOD FOR COUNTING THE NUMBER OF FUNGI IN THE SOIL<sup>1</sup>

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The numbers of fungi in the soil are usually determined by the plate method used for the determination of the number of bacteria. In view of the fact that the dilution used for the determination of bacteria is necessarily high, due to the large numbers of bacteria in the soil, the fungi are so diluted that very few appear on the plate: of a dozen plates prepared from the same soil, using the same dilution, three or four may be free from fungi entirely, three or four may have only one or two fungus colonies, while three or four may have several colonies, particularly in the case of humus-rich and acid soils. It has been pointed out by the author (1922 a) that the probable error involved in the determination of the numbers of fungi by this method is so great, as to make the results absolutely worthless.

To reduce the variability of the numbers of fungi on the plate and thus obtain a low probable error, low dilutions have to be used, so as to have 30 to 100 fungus colonies developing on the plate; this would necessitate a dilution of only 500 to 2000 for an ordinary fertile soil. But, if the common plate used for the determination of bacterial numbers is employed, so many bacteria will develop on the plate, as to prevent the development of most of the fungi.

To obviate this difficulty, use was made of the fact that fungi can grow readily at a much higher acidity than the bacteria and actinomycetes.

The author and others have long made use of the fact, that, when a culture of a fungus is wanted free from bacteria raise

<sup>1</sup>Technical Paper No. (61) of the New Jersey Agricultural Experiment Station, Department of Soil Chemistry and Bacteriology.

agar, which is acid in reaction may be used, or a drop of lactic acid added to each tube of the common media.

A medium has therefore been devised, having a reaction acid enough to prevent the development of the actinomycetes and the great majority of bacteria. At first, raisin agar was used, but, in view of that fact that this medium is not definite in composition and its reaction depends on the acid content of the raisins, the following synthetic medium has been developed.

Glucose.....	10	grams
Peptone.....	5	grams
KH <sub>2</sub> PO <sub>4</sub> .....	1	grams
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.5	grams
Distilled water.....	1000	cc.

Dissolve by boiling, add enough  $\frac{N}{1}$  acid (H<sub>2</sub>SO<sub>4</sub> or H<sub>3</sub>PO<sub>4</sub>) to bring the reaction to a pH = 3.6 to 3.8. This will require from 12 to 15 cc. of N acid per liter of medium. Add 25 gm. of agar, dissolve by boiling, filter, tube and sterilize as usual. The final reaction should be pH = 4.0.

The soil is now diluted, in the regular way, to only  $\frac{1}{50}$  to  $\frac{1}{200}$  of the highest dilution used for the determination of bacteria and plates are prepared in the regular way. The plates are incubated for seventy-two hours at 25°C. To obtain an accurate count and a low probable error, 10 plates should be prepared. The colonies may be counted after 48 hours, then after 72 hours, due to the fact that in some soils, rich in mucorales, the spreading forms will tend to overgrow the plate in 72 hours.

The following table gives a comparison of the numbers of fungi obtained from the same soil by the regular bacterial plate and the special method hereby suggested. A cultivated field soil was used for this purpose.

Instead of an impossible figure of 460,000 fungi per gram of soil, only 29,400 have actually been found by the modified method. The variability of the common method is so great as to make it valueless. Further details on the application of the new procedure will be published elsewhere (1922 b).

This method can also be applied to the determination of the number of fungi (molds) in various food preparations.

TABLE 1

	SOIL DILUTION	
	200,000	1000
Medium.....	Egg-albumen agar (used for the determination of bacteria)	Special medium
Number of colonies.....	2	31
	1	40
	5	40
	0	24
	7	38
	2	22
	1	24
	3	22
	0	20
	2	30
Mean.....	2.3 ± 0.47	29.4 ± 1.70
$\sigma$ .....	2.21 ± 0.33	7.97 ± 1.19
C.V.....	96.1 ± 14.3 %	27.1 ± 4.0 %
Em.....	20.4%	5.8%
Number of fungi per gram of moist soil....	460,000 ± 94,000	29,400 ± 1,700

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# STUDIES ON THERMOPHILIC BACTERIA

## I. AEROBIC THERMOPHILIC BACTERIA FROM WATER<sup>1</sup>

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### I. INTRODUCTION

The discovery of microorganisms that are able to live at relatively high temperatures (60°C. and above) has forced us to change our ideas on the resistance of protoplasm to heat and to admit that life is possible above the generally fixed limit of 42° to 45°C. The term thermophilic was probably first used by Miquel (1879) to describe those organisms that grow at temperatures so high as to be fatal to most microorganisms. This conception seems to have been lost sight of by many more recent workers. In order to have a better understanding of what the term thermophilic means, a number of definitions of the term as found in different texts on bacteriology are included.

In his physiological classification of bacteria, Giltner designated as thermophilic those that have a minimum temperature of 45°C., optimum, 55°C. and maximum, 70°C. Muir and Ritchie define thermophilic bacteria as organisms that grow best at a temperature of from 60° to 70°C. Hiss and Zinsser say that thermophilic bacteria are high temperature bacteria obtained from hot springs and from the upper layers of the soil. Rahn in Marshall's Microbiology describes thermophilic bacteria as extraordinary organisms having their maximum between 70° and 80°C., a temperature which coagulates albumin; corresponding to the high

<sup>1</sup> Abstracted from a thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Bacteriology. Copies of the original thesis are on file in the Library and Bacteriology Seminar of the University of Illinois.

maximum the thermophiles have a very high optimum, and the minimum lies with most species above 30°C. According to Hewlett there is a group of so-called thermophilic bacteria which thrive best at a temperature of 60° to 70°C. Those bacteria whose optimum temperature is above 40°C. and which are spoken of as the "thermophil" bacteria, is the definition given for them by Morrey. Buchanan does not mention thermophiles in his book but speaks of the organisms which produce large quantities of heat as thermogenic bacteria. In his book Chester places thermophilic bacteria in a class that does not grow at room temperatures or below 22° to 25°C.

## II. RELATION OF HIGH TEMPERATURES TO LIFE

All living things have their minimum, maximum and optimum temperatures for growth and other functions. The range of temperatures at which they exist may depend among other factors, on the species and on the ancestral history of the individual. Many investigators have experimented on the growth of organisms at high temperatures with varied and interesting results.

The first data concerning organisms that live at high temperatures were published by Sonnerat (1774). He reported on fish that lived in water at a temperature of 69°C. Schwabe (1837) reported the growth of algae in a hot spring at Karlsbad at 70°C. Flourens in 1846 mentioned algae which flourished in a hot spring at a temperature of 98°C. Brewer (1866) found some "Nostoc-formen" in a hot geyser at 83°C. Ehrenberg reported the existence of red and green algae from the Island of Ischia which grew at 63° to 65°C.

## III. HISTORICAL

The literature on thermophilic microorganisms has already become quite voluminous and in order to save space in this publication, we have summarized in table 1, those papers which a careful search of the literature has revealed.



## IV. EXPERIMENTAL

*Sources of cultures.* The present investigation has been limited to a study of some of the characteristics of 52 cultures of aerobic thermophilic bacteria from water; a more detailed study of forms from other sources is now being made. The cultures of thermophilic bacteria used in this investigation were isolated from samples of water furnished by the Illinois State Water Survey. Permission to use the samples was obtained from Professor E. Bartow, while director of the Water Survey. These samples came from different places in the state of Illinois and had been collected and shipped to the Water Survey according to directions furnished by them.

Many types of waters from different sources including deep wells, shallow wells, drilled wells, dug wells, springs, raw and treated municipal supplies and springs were used. In this manner it was possible to carry on a more representative study than if the samples had been taken from a restricted area. Out of 224 samples of water, 60 were found to contain thermophilic bacteria according to the method adopted for their isolation. This is to be regarded as a minimum for it is believed that thermophiles are quite abundant in nature and many samples which were negative when 1-cc. portions were examined would probably have been positive had a larger amount been used.

*Method of isolation.* Agar plates were poured in the usual way using 1-cc. and 0.1-cc. portions of the samples; the plates were incubated at 55°C. for twenty-four hours. Most of the thermophiles grow very rapidly at 55°C. and a longer incubation period was unnecessary. Any colonies that had developed in that length of time were transferred to agar slants; later it was found that it was easier to keep the cultures in broth since agar dried so quickly at 55°C. Control plates and agar plates that had been exposed to the air of the laboratory were incubated under the same conditions, but in no case was there any growth of thermophiles shown, either from the agar itself or from the air of the laboratory.

TABLE I

INVESTIGATOR	ORGANISMS DESCRIBED	SOURCE	TEMPERATURE	REMARKS
Miquel (1879-88)	<i>Bacillus the morphilus</i>	Seine River water; sewage excreta; dust; air	42°-70°C. 65°-70°C in air	Attributes the property of this organism to grow at such high temperature to particular character of protoplasm. Isolated first in 1879; characteristics described in 1888
Van Tieghem (1881)	(1) Streptococcus (2) Bacillus	Water in which beans had been cooked	Up to 74°C. Up to 77°C.	A certain amount of acidity produced by these organisms soon rendered the media uninhabitable by them
Cortes and Carrigou (1886)	(1) Small rods (2) Filaments	Hot spring at Luchon	45°-61°C.	"Further experiments are necessary to determine the chemical and biological action of these rods, and this knowledge will throw a light on the therapeutics of mineral waters."
Globig (1888)	Many bacilli (30 kinds)	Garden soil	50°-70°C.	Thought the fact that most of them were isolated from the superficial layers of the soil explains that they get heat for high temperature at which they grow from the rays of the sun. Source: not intestinal tract of man or animals, tap or river water
Burrill (1889)	Two bacilli	Silage; manure	60°-70°C.	The initial high temperature which these bacteria induce is probably most serviceable by causing the closer packing of the silage and the exclusion of the air, rather than by killing the germs of other ferment
Schlossing (1889)		Stable manure	Up to 79.5°C.	At temperature of 60°-66°C. these organisms produce 17 times as much carbonic acid as that in sterilized manure
Cohn (1893)				Attributed to thermogenic bacteria a rôle in so-called spontaneous heating of malt, tobacco leaves, cotton, hay, and manure. Made no attempt to isolate any of these cultures by the plate method

Flügge (1884)	Many bacteria	Sterile milk	24°-44° C. or 27°-54° C.	All strongly peptonizing in character; some were toxic; all formed spores which would withstand heating in water or steam for two hours
Leichman (1894)	A bacillus	Slimy milk	45°-50° C.	Produced acid
MacFadyen and Blaxall (1894)	Many bacilli	Earth, river and sea water, river mud, air dust, straw and feces of men, mice and chickens	60°-65° C.	"Their most marked property appears to be the decomposition of proteid bodies which they are able to effect." Most of them possess active fermentation properties
Gorini (1895)		Milk	37°-	Ambróz (1910) suggested that this organism was thermotolerant since it grew at 37° C. also
Karlinski (1895)	(1) <i>Bacillus Illdzensis capsulatus</i> (2) <i>Bacterium Ludwigi</i>	Hot springs of Hlbeze in Bosnia	50°-58° C. 55°-57° C.	Has no explanation to offer as to significance of the presence of these two organisms in hot springs. Suggests that the examination of other hot springs for similar bacteria would greatly increase the knowledge of biology of water bacteria
Rabinowitsch (1895)	Eight species of thermophilic bacteria	Many sources; widely distributed in nature	54°-75° C.	Concluded that peculiar ability of so-called thermophilic bacteria to grow at temperatures so much higher than the optimum temperature for common bacteria is a property of adaptation to environment
Weber (1895)	Bacillus I Bacillus II Bacillus III	"Sterile" milk	22°-60° C. 22°-60° C. 30°-65° C.	Found thermophilic bacteria in eight out of eleven samples of so-called "sterile" milk. Two of the three bacilli formed spores; none liquefied gelatin; all produced hydrogen sulphide rapidly
Harding (1896)	Two or three kinds of thermophilic bacteria	Silage	50°-60° C.	No attempts made to characterize or identify the forms found

TABLE I—Continued

INVESTIGATOR	ORGANISMS DESCRIBED	SOURCE	TEMPERATURE	REMARKS
Kedzior (1886)	Cladotrix form	River Spree	36°-70°C.	Facultative anaerobe grows better without oxygen. Spores very resistant to heat and to disinfectants such as 5 per cent phenol
Teich (1896)	Bacillus form	Hot spring of Illitze	51°-58°C.	Large oval spores formed in one end of the rods make them appear club-shaped
Davis (1897)	Probably a bacillus	Hot springs of Yellowstone Park	Up to 85°C.	Functions in the formation of mineral deposits in hot springs
Harshbarger (1897)	White filamentous bacteria	Hot springs of Yellowstone Park	85-4°C.	Becomes of a sulphur yellow color at 175°F. Yellow growth due to species of Beggiatoa, a plant which is classed with the Bacteriaceae, and which, during life, deposits sulphur granules
Koning (1897)	<i>B. tabaci</i> III <i>B. tabaci</i> IV <i>B. tabaci</i> V	Tobacco		Ambroz (1910) speaks of these organisms as real thermophilic bacteria
Miyoshi (1897)	Many bacillus forms; a zooglia masses of bacteria, iron bacterium	Hot springs in Japan	41°-69-8°C.	Hot springs of Japan furnish a good medium for thermophilic bacteria
Wittins (1897)		Hot springs in Switzerland		Ambroz (1910) explains the negative results of Wittins on the grounds of inadequate methods of research
Lynn (1898)	<i>Clostridium gelatinosum</i>	Fullmase in sugar manufacture	25°-58°C.	This organism is a facultative anaerobe whose spores are not killed by exposure to steam at 100°C. for 15 minutes

Opresecu (1898)	<p><i>Bacillus thermophilus liquefaciens aerophilus</i>  <i>Bacillus thermophilus aerobius</i>  <i>Bacillus thermophilus aquatilis</i>  <i>Bacillus thermophilus roticens</i>  <i>Bacillus thermophilus liquefaciens tyroenus</i></p>	Soil, Berlin Zoological gardens Canal water Spring water, ice Blood serum test glass Roquefort cheese	21°-70°C. 36°-60°C. 36°-60°C. 36°-62°C. Room temperature to 60°C.	Opresecu gave rather a detailed description of the five forms studied by him
Poupe (1898)	An organism similar to <i>Clostridium oclatinosum</i> Laza (1898)	Syrup	45°C.	After experiments on bacteria from soil carried on at different temperatures, he came to the conclusion that thermophilic bacteria are not properly so-called; the term thermotolerant should be applied to those organisms which can adapt themselves to high temperatures
Schillinger (1898)	Four types	Soil	60°C. max.	
Tsklinsky (1898 and 1899)	<i>Thermoactinomyces vulgaris</i> <i>Thermomyces lanuginosus</i>	Soil	48°-68°C.	Also isolated 6 varieties of bacteria from the hot springs on Island of Ischia which she called strict thermophiles; optimum temperature 60°C.
Michaelis (1899)	<i>B. thermophilus aquatilis liquefaciens</i> <i>B. thermophilus liquefaciens aerobius</i>	Spring water, Berlin	56°-60°C. optimum	Michaelis said of these organisms that they were not only thermotolerant, but also thermophilic
Vernhout (1899)	<i>B. thermophilus aquatilis chromogenes</i> <i>B. thermophilus aquatilis angustioris</i>	Fermenting tobacco at 44°-50°C. Water, feces, soil, pus, milk, etc.	25°C. optimum 50°-70°C. optimum	Not a true thermophile since its optimum temperature was 25°C. Sames suggests that a distinction be made between "thermophilic" and "thermotolerant" bacteria
Sames (1900)	Nine organisms not named			

TABLE 1—Continued

INVESTIGATOR	ORGANISMS DESCRIBED	SOURCE	TEMPERATURE	REMARKS
Dupont (1902)	<i>Bacillus thermophilus</i> Grignonii	Manure	50°C.	Also found <i>B. subtilis</i> and <i>B. mesentericus</i> rather a few times at this high temperature. The organism was proteolytic in character
Russell and Hastings (1902)	A micrococcus	Pasteurized milk	20°-25°C. optimum 76°C. maximum	Not a true thermophile but probably a thermotolerant organism
Schardinger (1903)	Group I—6 varieties Group II—I aerobic and 5 anaerobic organisms	Foods, etc. Foods, etc.	Room—35°C. 37°-66°C.	Grouped with hay and potato bacilli. Produce NH <sub>3</sub> from nitrates. Found that these bacteria "dehydrate" starch
Setchell (1903)	Filamentous Schizomycete	Hot springs of Yellowstone Park	70°-89°C.	Such bacteria grow at a higher temperature in silicious than in calcareous waters
Tsikhinsky (1903)	Eighteen bacilli. Two streptothrix forms	Human alimentary tract	57°C.	Believed the constant appearance of thermophilic bacteria in feces was explained by their wide distribution in nature and their great resistance; thermophiles are probably merely variations of common non-thermophilic organisms
Catterina (1904)	<i>Bacterium thermophilus radiatus</i>	Water	60°-70°C. optimum	Also isolated an organism apparently identical with <i>B. thermophilus</i> IV (Rabnowitsch, 1895)
Gilbert (1904)	<i>Actinomyces thermophilus</i>	Soil	50°-55°C. optimum	Strict aerobic; liquified gelatin slowly; coagulated milk
Kehler (1904)	Two forms	Hot spring	65°C. optimum	An anaerobic gram positive organism which produced large central spores
Benignetti (1905)	A bacterium	Hot spring	60°-75°C. optimum	
Brauni (1905)	Thirteen bacilli, five streptothrix forms	Adult and infant stools		Four bacilli and one streptothrix absolute thermophiles; all strict aerobes; all gram positive; all but one spore formers

	<i>Bacillus thermophilus</i> alpha	Hay	40°-70°C.	Hay in which spontaneous combustion had occurred
Miehe (1905)		Hay	40°-70°C.	Only occasionally found thermophilus in human alimentary tract
Anitschkow (1906)			59°-60°C.	
Bardou (1906)	<i>Bacillus thermophilus</i> alpha <i>Bacillus thermophilus</i> beta <i>Bacillus thermophilus</i> gamma <i>Bacillus thermophilus</i> delta	Sewage	59°-60°C. optimum	Ambrós says Bardon's work on the chemical reactions of thermophilus is fundamental and far surpassed in detail and accuracy other work that had been done. Three of organisms described were denitrifiers; all of them were strongly proteolytic
Blau (1906)	<i>B. cylindricus</i> <i>B. robustus</i> <i>B. loutus</i> <i>B. calidus</i>	Soil	60°C. optimum	Thermal death point 100°C. for 29 hours Thermal death point 100°C. for 7½ hours Extremely resistant spores. Thermal death point 100°C. for 19-29 hours Thermal death point 100°C. for 8 hours Regarded thermophilic bacteria of sanitary significance in water
Brazzola (1906)				
Falcioni (1907)	<i>B. thermophilus</i> I <i>B. thermophilus</i> II <i>B. thermophilus</i> III	Hot springs	60°C. optimum	Concluded that hot springs form favorable media for thermophilic bacteria
Miehe (1907)	<i>B. califactor</i>	Hay	59°-60°C. optimum	Claimed this organism was responsible for heating of hay. Called an orthothermophile. (Maximum above coagulation point of protein, 60°-70°C.)
Tirelli (1907)	Four rods; four cocci; two thread forms	Drinking water	55°-65°C. optimum	Believed temperature relations due to particular chemical nature of protoplasm rather than to their adjustment to the circumstances of their environment
Schütze (1908)	Many forms of <i>B. califactor</i> (Miehe) type	Moist clover hay		Believed that thermophilus grew better under aerobic conditions at high temperature and at lower temperature they grew better under anaerobic conditions

TABLE 1—*Concluded*

INVESTIGATOR	ORGANISMS DESCRIBED	SOURCE	TEMPERATURE	REMARKS
Jäger (1909)				Discussed possibility of spontaneous combustion of different organic materials by thermophilic bacteria
Ambroz (1910)				A complete review of literature on thermophiles up to 1910
de Krayff (1910)	Ten rod forms	Soil, water, air in tropics	55°-65° C.	Claimed thermophiles were very abundant in tropical climates
Georgevitch (1910)	<i>B. thermophilus</i> Vranzensis <i>B. thermophilus</i> Jivioni <i>B. thermophilus</i> Losanitchis	Hot springs	56°-60° C. optimum 43°-45° C. optimum 72°-73° C. optimum	Proved thermophiles were very important in biological changes in nature
Koch & Hoffman (1911)	Two spore-forming bacilli One spore-forming bacterium One thread form	Soil	52° C.	Concluded that the nature of the media had a great influence on the temperature response of these organisms, since they grew in soil at a temperature of 28°-30° C. and would not grow on artificial media at this temperature
Barlow (1912)		Canned corn		An organism said to be responsible for spoilage of canned corn
Kroulik (1912)				Thought bacteria and actinomycetes forms which decompose cellulose at 60°-65° C. are widely distributed in nature and occur specially where cellulose is naturally decomposed
Noack (1912)				Review of literature
Ambroz (1913)	<i>D-nitrobacterium thermophilum</i>	Soil	37° C. below minimum temperature	Called a true thermophile because it would not grow at room temperature nor at 37° C. Ambroz thinks the importance of thermophilic bacteria in the cycles in nature can not be overestimated since they play such an important rôle in metabolic processes



Negre (1913)	Numerous forms	Sand of Sabara	Studied decomposition of cellulose by thermophilic bacteria
Pringsheim (1913)			
Kosowicz (1912-13)			
Bergsy (1919)	Nine different bacteria	Dust, soil, etc.	Studied thermophilic bacteria in sugar juices
Cheney (1919)	Organisms like I, VI, and VI of Rabinowitsch	Canned foods	Suggested separating into two groups, the true thermophiles and the facultative thermophiles
Patzschke (1919)	<i>Streptococcus lacticus thermophilus</i>	Milk	Explained fact that so few thermophiles are found in canned foods on the market by supposing that nearly all cans containing them become spoiled and so are automatically eliminated before reaching the market
Weinzirl (1919)	<i>B. thermoindifferens</i> <i>B. aerothermophilus</i> <i>B. thermoalimentophilus</i>	Canned foods	
Donk (1920)	<i>B. stearothermophilus</i>	Canned corn; string beans; corn on cob	Found in corn that had been processed at 118°C. for 75 minutes
Bigelow and Esty (1920)			Studied the thermo-resistance of spores of thermophilic bacteria. Found that the hydrogen ion concentration of the media had a definite influence. Used term "obligate thermophiles"
Grieg-Smith (1920)	Spore-bearing rod	Fermenting tan-bark	May live at 80°C. in the fermenting tan-bark stacks

*Methods of study.* Inoculations into the different media used in this work were made either from twenty-four-hour agar slant cultures or twenty-four-hour broth cultures. Since this work was begun before the adoption of the new chart, the Descriptive Chart of the Society of American Bacteriologists indorsed in 1914 was used in the study of these thermophiles. The group number for each culture was determined under as uniform conditions as possible. In the work that is now under progress on thermophilic bacteria from soil, canned foods, and other sources the Descriptive Chart indorsed by the Society of American Bacteriologists at the meeting of December 30, 1920, will be used. The index number, it is believed, will give a better description of the organisms since it seems to embody characteristics which are more important.

*Media and technic.* With one or two exceptions the media and technic used in this study were those recommended by the Committee on the Descriptive Chart of the Society of American Bacteriologists in their report on Methods of Pure Culture Study (1920); the cultures were, however, all grown at 55°C. Other exceptions will be mentioned later.

*Morphology.* All the cultures studied were motile rods and usually grew in chains containing from two to many individuals. Sometimes chains of four or five rods showed an active snake-like movement. The rods were both long and short; some had rounded ends. Carbol fuchsin and Gram stains were used to stain the smears; all were Gram positive except nos. 10, 20, 40. Without exception, the cultures studied formed spores. Some of the spores were central and some polar; some were oval and some round; in a few cases the diameter of the spore seemed to be larger than that of the rod and produced a sort of clostridium form.

*Nutrient broth.* Witte's peptone was used in the nutrient broth employed in this study, since it seemed to possess certain advantages over other peptones. Good growth was secured with all strains in nutrient broth at 55°C. Most of the cultures produced turbidity and sediment in the broth; the surface growth in many of the cultures was membranous or showed a heavy pellicle.

*Indol.* Tests for indol were made on nutrient broth cultures that had been incubated for four days at 55°C. Both Ehrlich's test and the Nitroso-indol test were used. It was found that Ehrlich's test was much more satisfactory when the tubes were heated slightly. All of the cultures formed indol from Witte's peptone in varying amounts.

*Hydrogen sulfide formation.* To determine hydrogen sulfide formation, nutrient broth (made of Witte's peptone) over which a strip of lead acetate paper was suspended by means of the cotton plug, was used. The cultures were incubated for four days at 55°C. The blackening of the paper indicated hydrogen sulfide formation. All the cultures studied formed H<sub>2</sub>S. Streak cultures on "Bacto Lead Acetate Agar" plates also showed that all the cultures formed H<sub>2</sub>S. This latter medium seemed to be well adapted to the determination of this characteristic.

*Potato slants.* The growth of these thermophiles on potato slants was abundant in most cases even after twenty-four hours at 55°C. The type of growth varied from a filiform to spreading growth. The potato was turned gray, brown, or reddish brown. The cultures could not be kept longer than from two to three days since they dried out so quickly at 55°C., but the growth at this temperature was quite rapid on this and other media.

*Liquefaction of gelatin.* The "provisional method" was used to determine this characteristic. The cultures were first accustomed to the gelatin medium by preliminary cultivation for eighteen to twenty-four hours in a 1 per cent solution of gelatin at 55°C.; then the surface of gelatin in test tubes was inoculated and the tubes incubated for thirty days at 20°C. All the cultures except nos. 6, 15, 20, 32, 51 had partially or completely liquefied the gelatin at the end of thirty days. Gelatin cultures prepared in the same way and incubated at 55°C. for four days were all liquefied with flocculent growth throughout the gelatin and would not harden when placed in the refrigerator. The fact that all the cultures studied liquefied gelatin at 55°C. and some of them at 20°C. can probably be explained by the fact that 20°C. was below the minimum temperature for growth for those cultures which did not liquefy gelatin at that temperature.

*Litmus milk.* In this medium azolitmin was used as the indicator. The litmus milk cultures were incubated at 55°C. for four to seven days, only, because they evaporated so quickly at this temperature. Peptonization occurred with at least 75 per cent of the cultures; and in each of these cases the medium was alkaline. All of the cultures curdled the milk and in those cases where peptonization did not occur the medium was acid. More work is being done on the growth of these organisms in milk and particularly on the use of brom-cresol purple as an indicator.

*Fermentation of sugars and glycerol.* No gas was formed by any of the cultures. None of the cultures produced acid in lactose; the cultures varied slightly in their formation of acid in glucose, sucrose and glycerol broth. Brom thymol blue was used to test for acidity since that indicator was used to adjust the reaction of all the broths when made. The amount of acid formed in the different broths by these cultures may be of significance and should be determined.

*Oxygen relation.* This characteristic was determined by noting the presence or absence of growth in the open and closed arm, respectively, of fermentation tubes containing glucose broth. All the cultures used in this study were found to be strict aerobes.

*Reduction of nitrates.* To determine this characteristic, both nitrate broth and nitrate agar slant cultures incubated at 55°C. for four days were used. Sulphanilic acid and alphanaphthylamine were used to test for nitrites. All of the cultures reduced nitrates.

*Diastatic action on starch.* Two per cent agar containing 0.2 per cent of soluble starch was used for this determination because this stiffer agar seemed to stand the incubation at 55°C. better. Dot inoculations were made in the center of the petri dishes containing the hardened starch agar; these were incubated at 55°C. for forty-eight hours since longer incubation dried the agar and made it crack. All the cultures grew well on this media and all produced diastatic action, some feeble and some strong.

*Temperature relations.* Some of the cultures were grown on agar slants at different temperatures and it was found that 50° to 55°C. was the optimum temperature for growth. Since it is

believed that the temperature relations of the thermophilic group of microorganisms is a subject worthy of intensive study, a separate investigation on this subject was initiated which is now nearing completion in this laboratory.

#### V. DISCUSSION

A comparative study of 52 strains of thermophilic bacteria from water indicates a group in which the characteristics are not widely divergent. All of the strains were spore formers and all liquefied gelatin. When separated into groups according to the "group number" they fell into nine groups. Most of these groups were defined by differences in the terminal reaction in glycerol and carbohydrate media. If these determinations are neglected all of the strains would have fallen into one group.

A survey of the literature on thermophilic bacteria indicates that many of the strains there described have been superficially studied and that new strains have been named without sufficient data. Consequently many of the names which are used for thermophilic bacteria are being applied to the same organism.

Without exception the 52 cultures which were used in this study formed spores and in this characteristic seem to agree with most of thermophilic bacteria which have been described in the literature. This then seems to be the most common characteristic of members of this group. It has also been the basis for including among the thermophilic bacteria, bacteria which do not belong there. Spore formation when taken into consideration along with the peculiar reaction to temperature makes the thermophilic bacteria a difficult group for canners of foods, for instance, to cope with. The ability to form spores allows the thermophilic bacteria to survive the process and perhaps to develop when the cans are stacked in the warehouse. The recent publications of Weinzirl, Cheney, Bigelow and Esty, and others have indicated the significance of these bacteria. They are also related to certain phases of the dairy industry. Flügge, Leichmann, Russell and Hastings have found that they are able to survive pasteurization temperatures.

The forms isolated from water were Gram positive and in this characteristic, also, agreed with the forms described in the literature. Their destructive nature is indicated by their action on the proteins in milk and on gelatin. Indol and hydrogen sulphide were produced in most media. Rabinowitsch (1895) reported the proteolytic abilities of the thermophiles to be their most characteristic function.

Although none of the cultures studied fermented any of the sugars used, many of them did produce some acid in glucose or sucrose broth; this together with the fact that all of them showed diastatic action on starch would seem to indicate that these thermophiles decompose the more complex carbohydrate molecules more readily than they do the simple sugars. That many of the thermophiles decompose cellulose quite readily is seen in a review of the literature on those types that function in spontaneous heating during the fermentation of malt, tobacco, cotton, hay, and manure, the fermentation of silage and the decomposition of cellulose.

The fact that some of these cultures came from water from quite deep wells and others from surface waters demonstrates that even in water the thermophiles can exist at widely varying ranges of temperature. They are probably widely distributed in waters and had larger portions of the samples of water been used for plating, no doubt, many more cultures would have been isolated from the 224 samples of water examined. The widely differing sources of the thermophiles described in the literature also indicate that they are widely distributed in nature and further investigations of their temperature relations will explain this distribution. Rabinowitsch (1895) and some others have claimed that this ability of thermophiles to grow at a high temperature was a property of adaptation to environment. Blau and Bruini both claimed that many non-thermophiles had many of the same characteristics as thermophiles. Other investigators have published data to support this claim. Bredfeld (1878) gradually developed the resistance of spores of *Bacillus subtilis* until it took three hours at 100°C. to kill them or five minutes at 110°C. Koch (1876) observed the germination of spores of *Bacillus*

*anthracis* and *Bacillus subtilis* that had been subjected to 123°C. in dry air. According to Arloing, Cornevin, and Thomas (1882) the spores of *Bacillus anthracis-symptomatici* would not resist boiling for more than 2 minutes; but if previously dried, boiling for two hours was necessary to destroy them.

This seems to agree with the theory of Davenport and Castle (1895) that by the loss of water, which is a necessary consequence of increased chemical activity resulting from warmth, organisms are able to increase their resistance to high temperatures. If we accept this view thermophiles are explained on the basis of adaptation to environment.

Tsiklinsky (1903) also believed that thermophiles were merely variations of common non-thermophilic microorganisms that had adapted themselves slowly to high temperature. He thought that the length of time necessary for these organisms to adapt themselves to high temperature determined whether they were facultative or strict thermophiles.

Schillinger proposed the term thermotolerant to be applied to these organisms. Mische tried to explain the high optimum temperature of thermophiles by assuming that they might have been brought over from the tropics and have adapted themselves to lower temperatures. By comparing them with other bacteria, Mische came to the conclusion that all bacteria could be grouped on the basis of their minimum temperatures. He divided the thermophiles into two groups: (1) orthothermophiles with a maximum temperature of 60°-70°C.; (2) thermotolerants with a maximum temperature of 50°-55°C. but which also grow well at ordinary temperatures.

Many investigators, among them Rabinowitsch, Schütze, and others, found a certain parallelism between temperature relations and the relation of thermophiles to oxygen. In most cases those organisms which had high optimum temperatures were strict aerobes.

Bergey divided thermophilic bacteria into two groups: (1) true thermophiles, those that grow at temperatures above the maximum temperature for the great majority of bacteria, especially the pathogenic forms; (2) facultative thermophilic

bacteria, those that develop at room temperature, about 20°C., and have their optimum temperature at about 50°C., and their maximum temperature at about 60°C.

It would seem to be indispensable to fix clearly the limits within which the term thermophilic bacteria should apply. Some division evidently must be made in this group of organisms that grow at such widely differing ranges of temperature. The division made by Bergey into true thermophiles and facultative thermophiles seems to be the most tenable up to the present time. Further work on temperature relations of these thermophiles is being carried out in this laboratory and perhaps when the data from this investigation are available a better differentiation will be possible.

Of the 52 waters from which thermophilic bacteria were isolated 44 (almost 85 per cent) were condemned for the presence of *B. coli* of fecal origin. This fact suggests a possible sanitary significance of thermophilic bacteria in water analysis. The data are insufficient to draw any definite conclusions on this subject but it is a subject worthy of investigation. A similar suggestion was made by Brazzola (1906) when he stated that he thought the thermophiles were of very great importance in the study of the potability of water.

#### VI. CONCLUSIONS

1. The aerobic thermophilic bacteria studied in this investigation seemed to make up a closely related group when the salient characters only are considered.
2. All strains form spores and are strongly proteolytic which, in connection with their temperature relations, makes them of importance in food preservation.
3. Thermophilic bacteria are widely distributed in nature (soil, water, etc.) and thus may cause serious losses in those industries where high temperatures are used for controlling bacterial development.
4. The ability of thermophilic bacteria to grow at high temperatures may be due to a particular property of the protoplasm (water content?).



5. Further investigations on their temperature relations may aid in a better understanding of the thermophilic bacteria and in their separation into more sharply defined groups. This work is in progress in these laboratories.

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# AN APPARATUS FOR THE RAPID MEASUREMENT OF SURFACE TENSION<sup>1</sup>

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Investigations carried on in these laboratories concerning the rôle of surface tension in certain bacteriological phenomena (Larson, 1921), have led to the development of an apparatus for the rapid determination of surface tension by the drop-weight method. After using the more common methods of surface tension measurement the belief is expressed that the most reliable and constant results for both pure and biological liquids can be obtained from their drop weights. The apparatus here described represents a means of determining the surface tension of a liquid by the drop-weight method without any mathematical calculation or the determination of the drop weights and is termed a surface tension balance. The apparatus is designed primarily for rapidity of measurement and an accuracy is obtained which is consistent with ordinary experimental conditions.

In general the apparatus consists of three mechanical parts, a dropping pipette, a balance beam mounted on a torsion wire and an adjustable scale.

The dropping pipette (A) differs in no way from those already in use and all the inaccuracies and corrections, which have been recorded for this method, occur but such errors may be minimized and reduced to an order of magnitude which is of no concern to the biologist, by using a dropping surface of the proper dimensions (Morgan, 1911). The rate at which the drops fall may be easily controlled by using a fine capillary tube for an air inlet protected by larger tubing (I).

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The steel torsion wire (*D*) is tightly stretched between two binding posts and carries the balance beam, one end of which (*B*) swings over the face of the adjustable scale (*F*), the opposite end carrying a silver cup (*C*) swung on watch jewels. The balance beam can be swung into the horizontal or zero position by means of the bar (*H*).

The adjustable scale consists of a series of arcs of the same radius but the segments are of different lengths and each segment is divided into the same number of units by cross lines. The scale face (*F*) is movable horizontally on the scale carrier (*E*) in the visible slot and is fixed in any position by means of a thumb screw behind. This mobility allows any one of the various sized arcs to be moved directly under the end of the pointer (*B*). The scale is constructed in relation to the size of the torsion wire so that the units of the scale represent surface tension in dynes per centimeter.

The weight of a drop of liquid falling from the pipette (*A*) into the cup (*C*) will be proportional to the surface tension of the liquid according to the formula.

$$\gamma = \kappa\omega$$

where  $\gamma$  = surface tension

$\omega$  = weight of drop

$\kappa$  = constant of dropping pipette.

The force producing torsion in the wire will be the drop weight times the acceleration of gravity and the amount of torsion produced will be proportional to this force. The degree of torsion indicated by the pointer is therefore directly proportional to the surface tension of the liquid dropping from the pipette. The mobility of the scale makes it possible to divide any arc indicated by the pointer into a desired number of units by moving under the end of the pointer the proper sized scale arc and in this way an arc of torsion that is proportional to a surface tension of sixty dynes may be divided into sixty units and then each unit will represent a dyne of force.

The surface tension balance is calibrated by means of a standard liquid of high surface tension as follows: A drop of the liquid of known surface tension is dropped from the pipette into the cup.



The pointer then swings into some position over the face of the scale. The scale face is then moved horizontally until the reading off the end of the pointer is the same as the surface tension of the liquid used in calibrating. The balance is then ready for use and if a liquid is now used which has one half the surface tension, the weight of the drop will be one-half, the torsion produced in the wire will be one-half and the reading on the scale will be one-half the previous reading.

The sensitiveness of the apparatus varies with the mechanical construction depending upon the ratio of the mass of the balance beam to the torsion constant of the torsion wire. The number of drops of liquid used in determinations can vary from a single drop to many if a torsion wire of the proper size be chosen. Five drops have been found very satisfactory in our work.

Evaporation is the greatest source of error with this apparatus. It cannot be used for very volatile liquids. However, if calibrated with water and used for watery solutions, the error from evaporation is rendered negligible in that under like experimental conditions, the same evaporation takes place in calibration that occurs in actual measurements. This has proven out in that repeated runs of watery solutions give identical results to the limit of readability of the scale. The temperature variation may be rendered negligible by controlling temperature or proper corrections may be made. The accuracy of the surface tension balance now in use is plus or minus one-tenth dyne.

The pipette is the only part of the apparatus to be cleaned as the cup need only be dried and the pointer reset to zero. The pipette may be cleaned very rapidly if suction and compressed air are available. Since the time consuming weighings are eliminated the measurements may be taken with great rapidity. With this apparatus the surface tension of thirty solutions were measured in one hour.

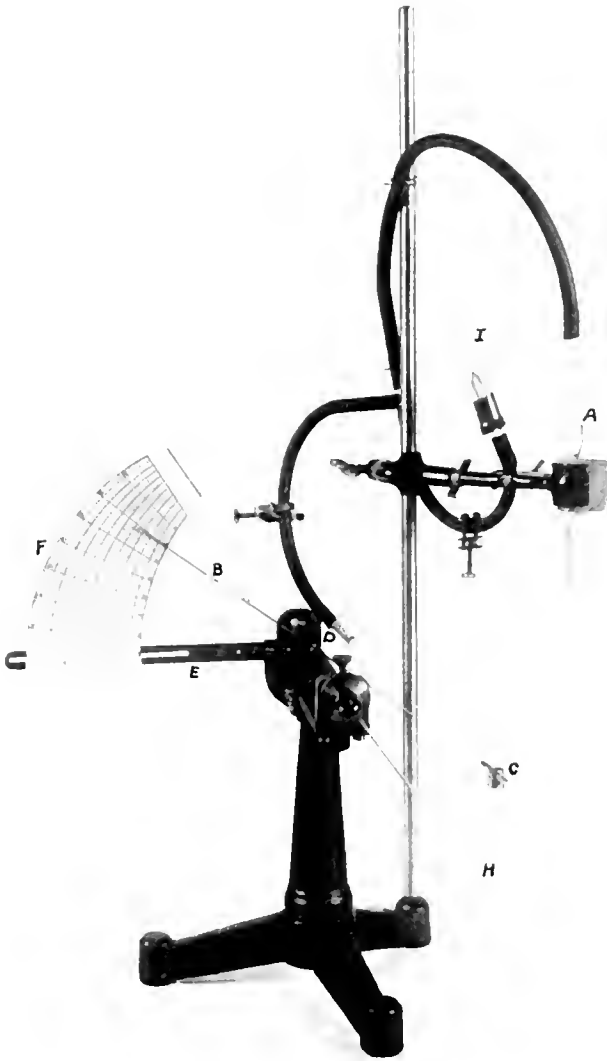
#### SUMMARY

An apparatus termed a surface tension balance is described for the rapid measurement of surface tension by the drop-weight

method. The apparatus consists of a dropping pipette, a delicate torsion balance and an adjustable scale upon which the surface tension is read directly in dynes per centimeter.

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(Green: Rapid Measurement of Surface Tension)



# JOURNAL OF BACTERIOLOGY

## Contents

MARCH, 1922—Vol. VII—No. 2

- F. C. HARRISON. Our Society.  
VICTOR BURKE. Notes on the Gram Stain with Description of a New Method.  
BARNETT COHEN. Disinfection Studies. The Effects of Temperature and Hydrogen Ion Concentration upon the Viability of *Bact. coli* and *Bact. typhosum* in Water.  
SELMAN A. WAKSMAN. Microorganisms Concerned in the Oxidation of Sulfur in the Soil. I. Introductory.  
SELMAN A. WAKSMAN AND J. S. JOFFE. Microorganisms Concerned in the Oxidation of Sulfur in the Soil. II. *Thiobacillus Thiooxidans*, a New Sulfur-oxidizing Organism Isolated from the Soil.  
E. B. FRED AND W. H. PETERSON. The Production of Pink Sauerkraut by Yeasts.  
CONSTANTINO GORINI. Studies on the Biology of Lactic Acid Bacteria: A Summary of Personal Investigations.  
L. D. BUSHNELL. A Method for the Cultivation of Anaerobes.  
L. D. BUSHNELL. Influence of Vacuum upon Growth of Some Aerobic Spore-Bearing Bacteria.  
H. R. BAKER. Substitution of Brom-Thymol-Blue for Litmus in Routine Laboratory Work.  
W. A. WALL AND A. H. ROBERTSON. The Use of Domestic Methylene Blue in Staining Milk by the Breed Method.

JANUARY, 1922—VOL. VII—No. 1

- HILDA HEMPL HELLER. Certain Genera of the Clostridiaceae. Studies in Pathogenic Anaerobes. V.  
O. ISHII. Studies upon Agglutination in the Colon-Typhoid Group of Bacilli.  
O. ISHII. A Study of Spontaneous Agglutination in the Colon-Typhoid Group of Bacilli.  
ALBERT C. HUNTER. The Sources and Characteristics of the Bacteria in Decomposing Salmon.  
S. A. KOSER AND W. W. SKINNER. Viability of the Colon-Typhoid Group in Carbonated Water and Carbonated Beverages.  
GUILFORD B. REED. A Binocular Microscope Arranged for the Study of Colonies of Bacteria.  
An Investigation of American Stains.

NOVEMBER, 1921—VOL. VI—No. 6

- GEORGE E. HOLM AND JAMES M. SHERMAN. Salt Effects in Bacterial Growth. I. Preliminary Paper.  
HILDA HEMPL HELLER. Suggestions Concerning a Rational Basis for the Classification of the Anaerobic Bacteria. Studies in Pathogenic Anaerobes. IV. I. Preliminary Paper.  
J. HOWARD BROWN. Hydrogen Ions, Titration and the Buffer Index of Bacteriological Media.  
J. E. RUSH AND G. A. PALMER. On Decreasing the Exposure Necessary for the Gelatin Determination.  
HAROLD MACY. Chart of the Families and Genera of the Bacteria.

SEPTEMBER, 1921—VOL. VI—No. 5

- G. P. PLAISANCE AND B. W. HAMMER. The Mannitol-Producing Organisms in Silage.  
HILDA HEMPL HELLER. Principles Concerning the Isolation of Anaerobes. Studies in Pathogenic Anaerobes. II.  
JOHN F. NORTON AND MARY V. SAWYER. Indol Production by Bacteria.  
AUGUSTO BONAZZI. On Nitrification. IV. The Carbon and Nitrogen Relations of the Nitrite Ferment.  
TH. THJOTTA AND ODD FALSEN SUNDT. Toxins of *Bact. Dysenterias*, Group III.

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## CONTENTS

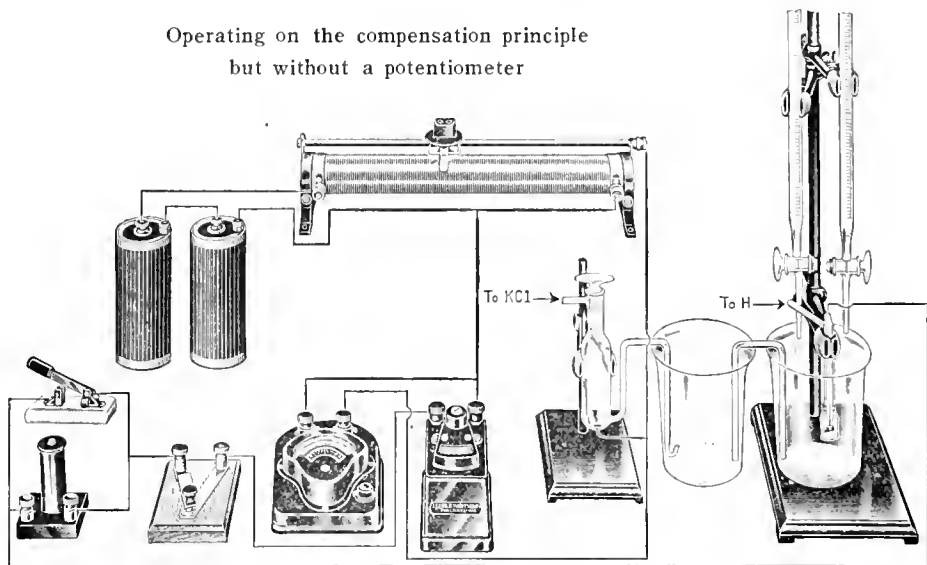
- L. D. BUSHNELL. Quantitative Determinations of Some of the Biochemical Changes Produced by a Saprophytic Anaerobe..... 373
- G. S. WILSON. The Proportion of Viable Bacteria in Young Cultures with Especial Reference to the Technique Employed in Counting..... 405
- H. J. CONN. A Method of Detecting Rennet Production by Bacteria..... 447

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# QUANTITATIVE DETERMINATIONS OF SOME OF THE BIOCHEMICAL CHANGES PRODUCED BY A SAPROPHYTIC ANAEROBE<sup>1</sup>

L. D. BUSHNELL

*Kansas Agricultural Experiment Station*

Received for publication December 3, 1921

## INTRODUCTION

The differentiation and classification of microbial species is certainly the most difficult of all problems in bacteriology. When one finds what he considers a new species he is at once aware of the lack of means to characterize it adequately so that it may be recognized by others. At the same time the similarities that it presents to other types, which are certainly distinct, may lead to confusion, when an attempt is made to place it properly among species already described. This confusion is more marked in the case of the anaerobic than of the aerobic types. This is probably due to the fact that anaerobic types are supposed to be very unstable in their characteristics and less care has been used in describing them; and also to the fact that many mixed cultures of anaerobes have been described as pure cultures.

Probably some of this confusion is due to the fact that too much emphasis has been placed on one factor, such as the form of the colony, or the location and size of the spore. It is a well known fact that colony formation is quite variable, in many cases being completely altered by the consistency of the medium. Again the location of the spore varies in many of the individuals of the same culture.

Bacteriological experience has taught us not to rely too much upon one factor, but rather to classify microorganisms according

<sup>1</sup> Contribution no. 41 from the Bacteriological Laboratories of the Kansas Agricultural Experiment Station.

29  
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to a group of factors. The fermentation of both carbohydrates and proteins should be considered, and a certain amount of cultural and morphological variation should be allowed for each group.

DESCRIPTION OF THE ORGANISM USED IN THE PRESENT  
STUDY

In the study of anaerobic bacteria isolated from spoiled canned asparagus we have found one type which predominated and which could be distinguished by colony formation in the deep agar column. This organism was a Gram positive, spore-bearing, anaerobic rod; motile, by means of 12 to 15 peritrichic flagella. The size of the majority upon plain agar was 0.80 to 0.90 by 2.50 to 3.50 microns. They were somewhat longer and more slender upon glucose media.

The surface colonies are very thin and transparent at first, but gradually assume a more opaque granular center from which the surface growth extends over the medium for several millimeters. The edge is quite regular in outline, slightly raised from the medium. The colonies later become more irregular with root-like projections extending from the edge of the colony while similar projections extend down into the medium below the colony. These colonies usually show a central nucleus which at first is more opaque than the remainder of the colony. The colonies are transparent but in most cases there is a whitish appearance in older cultures. Under low magnification the surface colonies appear to be finely granular but remain nearly colorless.

The deep colonies are spherical at first and more opaque than those on the surface. They later develop root-like outgrowths or may become quite woolly in some instances, especially if the medium is soft. Older colonies tend to become somewhat less opaque as they increase in size. Some of the colonies are somewhat lenticular in outline and have woolly or root-like outgrowths from one side only. This appearance is described as "*En grenade*" by Weinberg and Seguin (1918). Some of the cultures of this group appear ragged in outline, not having distinct projections but with a very irregular surface.

The organism would be considered to be proteolytic in its action upon protein-containing media. Gelatin is liquefied rapidly and completely. Inspissated serum is rapidly cleared, followed by nearly complete digestion. In alkaline meat media there is a rapid growth with the development of much gas and a distinctly unpleasant but not putrefactive odor. There is slight digestion of the meat at the surface. This, however, was not marked in any of the cultures. The surface layers of the meat are darkened, while the lower layers retain their original color. There is a heavy, whitish growth of bacteria on the surface of the meat in very old cultures. About 1 or 2 mm. below the surface is a blackened zone. Traces of hydrogen sulphide are found in meat cultures. The liquid remains neutral or slightly acid and the particles of fat on the surface appear to be partially disintegrated. Growth in Von Hible's brain media is very similar.

Growth in milk is rapid. There is very little or no gas formed. The casein is thrown down in a soft clot which is usually completely digested within four days. With some of the cultures, however, digestion is never complete. The whey separates and in a short time becomes of a clear light amber color, or yellowish and turbid, according to the culture. The reaction is neutral or slightly acid on standing, and the fat layer becomes disintegrated, probably due to saponification. The cultures have a strong cheesy to butyric acid odor. Some of the cultures are somewhat more putrid and upon long standing, especially if enclosed in a jar, there is a slight odor of ethyl-butyric or of valerianic acid. Cultures seem to differ considerably in this respect with age.

In the egg medium, made according to the formula given by Robertson (1916), the organism grows slowly. The first indication of growth is the appearance of blackening on the bottom of the tube. This is followed in some cases by the formation of a soft, yellowish clot which becomes fissured and may settle out, leaving a clear, yellowish, transparent fluid. Some of the cultures do not cause coagulation even on long standing, and those cultures which form a clot, fail to produce further change, except in a few cases, in which there is a marked shrinking of the clot. If the medium was made slightly acid nearly all the cultures caused

the formation of the clot, with darkening at the bottom of the tube; but aside from the shrinking no other change occurred.

Indol, phenol, skatol or alcohol were not found. There was a slight reduction of nitrates to ammonia. The pH as determined colorimetrically in Clark and Lubs media ranged from 5.8 to 6.2. Cultures were all negative for the methyl-red and the Voges-Proskauer reactions.

Considerable difficulty was encountered in determining which of the carbohydrates were actually fermented. The work was first done by the use of Durham's fermentation tubes, using meat infusion broth made sugar free by fermentation with *B. saccharolyte*. This medium was placed in tubes and autoclaved. To it was added 1 per cent of the carbohydrate to be examined and the tubes were incubated for the detection of contamination. Just before inoculation they were heated and cooled rapidly in running water. They were then inoculated with a large loopful of a four day potato-peptone culture of the organism. The cultures were placed over phosphorous in an anaerobic jar and incubated for three days at 37°C.

Difficulty was sometimes encountered, however, in determining the fermentation of such carbohydrates as lactose, inulin, etc., due to the fact that the closed arm of the tube would contain a bubble of gas from one to three millimeters in diameter. The reaction of the medium in these cases was but little changed, being sometimes made slightly acid. In other cultures, however, distinct amounts of acid were formed. Hiss's serum water plus these carbohydrates gave similar results.

Table I shows the essential features of these tests. The "+" indicates a distinct amount of acid or gas. If a bubble of gas is present it is marked with a "b."

From our preliminary tests it was decided that we were dealing with organisms belonging to the *B. sporogenes* group (Metchnikoff, 1908). There were differences between the various cultures, but these were not marked. Some cultures always produced a clouding of the medium, others grew mostly on the bottom of the tube. Certain cultures produced more blackening of the egg medium than others. Generally speaking all the cultures exhibited the same characteristics.



All the cultures were completely non-toxic when grown on brain and liver broth and on brain—saline for different lengths of time and injected subcutaneously into guinea-pigs in 1 cc. amounts. Table 2 shows the results of one series.

TABLE 2  
*Results of animal inoculation*

CULTURE	AGE OF CULTURE	DAYS TO DEATH OF ANIMAL
	<i>days</i>	
2 B	6	Found dead on 30th day (colon bacilli from heart blood)
5 B	16	Alive after 6 weeks
28 B	23	Alive after 6 weeks
37 B	34	Alive after 5 weeks

TABLE 3  
*Agglutination reaction*

RABBIT IMMUNIZED AGAINST CULTURE	CULTURE	DILUTION OF IMMUNE SERUM								NORMAL SERUM	
		1:25	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:50	1:200
2 B	2 B	*10	10	10	10	10	10	5	0	2	0
5 B	5 B	8	10	10	10	10	10	8	2	0	0
28 B	28 B	10	10	10	10	10	10	8	0	0	0
2 B	5 B	10	10	10	10	10	10	5	0	4	0
2 B	28 B	10	10	10	10	10	5	0	0	2	0
5 B	2 B	10	10	10	10	10	10	10	3	3	0
5 B	28 B	10	10	10	10	10	8	0	0	2	0
28 B	2 B	10	10	10	10	10	10	3	0	0	0
28 B	5 B	10	10	10	10	10	10	2	0	1	0
5 B	B. spor. I	10	10	10	10	10	9	3	0	3	0
5 B	B. spor. II	8	10	10	10	10	8	0	0	2	0
5 B	B. bot. II	10	10	10	5	0	0	0	0	2	0

\* Figures show amount of agglutination. 10 = complete.

Several of the cultures of this organism were also serologically similar to known cultures of *B. sporogenes*, as shown by table 3. Their non-toxic nature and the appearance of colonies in deep agar would differentiate them from *B. botulinus*, to which species they are otherwise very similar.



## QUANTITATIVE ANALYSIS OF BIOCHEMICAL CHANGES

Since the qualitative results left us in doubt concerning the fermentation ability of these organisms, we attempted to determine these points by quantitative tests.

Wolf and Harris (1917a; 1917b; 1918) and Wolf and Telfer (1917) and Wolf (1918; 1919) have conducted exhaustive experiments upon the biochemistry of pathogenic anaerobes from war wounds, using quantitative methods. These authors did not attempt to determine the ability of these organisms to ferment many of the carbohydrates ordinarily used for the separation of microorganisms into groups, and studied only those found in wound infection. They did, however, collect some very valuable information concerning some of the activities of a large number of the more common pathogenic anaerobes.

We hoped, by tests of this sort, to determine some more accurate and rapid method for the classification of anaerobes in general, and those in particular with which we were working. Since the dangers of botulism from the eating of canned foods is more prevalent than formerly, it is quite essential that bacteriologists should obtain all the information possible concerning the groups of spore-bearing anaerobic bacteria which may be found in food. As has been mentioned above, the classification of anaerobes is very much confused at the present time; and since their fermentative ability, as determined by the ordinary qualitative methods is difficult to determine accurately, the only apparent method of obtaining real differences is by quantitative methods.

We have, therefore, attempted to utilize the following quantitative indications of difference: (1) Amount and kind of gas produced from various carbohydrates; (2) amount and kind of acid produced from various carbohydrates; (3) amount of proteolytic action as determined by formation of ammonia and amino-acids.

The first condition to be fulfilled is to obtain a medium which will be as constant as possible in composition, and one in which organisms of this type are able to grow. The second condition

is to obtain an apparatus by means of which the various changes may be noted and recorded over a series of days, since the fermentations change with the age of the culture to a marked extent. We hoped to obtain more accurate information by this method although the amount of work necessary is multiplied in proportion to the number of determinations made.

Of course, it is impossible under all conditions to make the medium absolutely the same. However, we have attempted to do this as far as possible, by obtaining enough material in one lot for an entire series of tests. The same asparagus was used throughout; and for the carbohydrate fermentation tests, we used a 2 per cent peptone-water to which, after sterilization, was added 1 per cent of the carbohydrate in question, except in case of glucose of which 0.5 per cent was used. We used peptone-water rather than broth because this could be made more uniformly, and did not require an adjustment of the reaction. Unfortunately these organisms would not grow in any of the mineral solutions usually recommended for the culture of nitrogen fixing anaerobes, so their action in a medium of known composition could not be determined.

In their experimental work Wolf and Harris used a large 2-liter bottle for culturing the anaerobes. This bottle was fitted with a two-holed rubber stopper through which passed two glass tubes, one of the tubes extending into the space above the liquid and one into the liquid itself and nearly to the bottom of the bottle. The container was exhausted and the culture allowed to develop in vacuum until the space above the liquid was filled with gas. Samples were removed at intervals by means of the tubes. The presence of the gases was utilized to force out enough liquid for an analysis.

The apparatus described by these investigators could not be used by us, since our cultures did not grow very rapidly, and we invariably found that a leak had occurred and air had entered. Also, we experienced difficulty with contamination, in sampling according to their method. Aside from the difficulties mentioned, the apparatus recommended by Wolf and his associates would probably give more uniform results for a single series of examina-

tions than the one used in this work, since but one culture container was used and all the samples taken from it. Results could not be exactly duplicated, however, by the use of this apparatus.

All our cultures were placed in a vacuum as complete as we could obtain. Great difficulty was experienced in avoiding leaks, and in obtaining a culture vessel which would withstand sterilization and still be strong enough not to be crushed under the high external pressure. At the time this work was done, special glassware could not be purchased on the market and "pure" gum rubber tubing that would hold a vacuum was not obtainable. Numerous samples of so-called pure gum rubber, were secured, but none of them would hold a high vacuum for twenty-four hours, although they were coated with celloidin and various cements recommended for this purpose. We desired enough culture material for several tests and this required a large culture vessel.

#### DESCRIPTION OF THE APPARATUS

The following apparatus (fig. 1) was finally devised and met these difficulties very well.

A quart milk bottle (N) was used as a culture vessel. To this was added from 300 to 500 cc. of the culture medium and the bottle was then autoclaved at 20 pounds pressure for thirty minutes. The asparagus was merely suspended in tap water, 100 grams in 300 cc. of water. Fresh whole mixed milk was used. The potato medium was made by adding 100 grams of potato to 300 cc. of water. The potato was carefully selected, washed, peeled and passed through a meat grinder; and the potato pulp was washed in running water for several hours. For the carbohydrate fermentation tests a 2 per cent peptone solution was used. Three hundred cubic centimeters of this solution were placed in the bottles and sterilized. The carbohydrates were sterilized in 10 per cent solution and added at the time of inoculation.

As an inoculum, we used 1 cc. of a four day old potato-peptone-water culture of the organism grown at 37°C. The four day culture seemed to give the best results. At first smaller amounts

were used but in many cases we failed to obtain growth. Care was always used not to add bits of potato.

Just before inoculation, the bottles of media were heated in the steamer and then cooled as rapidly as possible to drive out the air. Care was always exercised not to shake up the medium during the inoculation and sealing process. After inoculation, the cotton stoppers were removed and the bottles were plugged with a No. 8, two-holed rubber stopper, fitted with two glass tubes. One of these tubes was constricted near the upper end and served as an attachment for the vacuum pump, and for sealing after the container had been exhausted. The second tube was bent to serve as a manometer tube (P) as indicated in figure 1. The rubber stopper was fitted with the tubes, wrapped in gauze and then covered with a thick layer of cotton. The lower end of the manometer tube and the upper end of the straight tube were plugged with cotton and all autoclaved at twenty pounds pressure for an hour. It is necessary to wrap the cotton stopper in the mouth of the bottle, and the rubber stopper with gauze to prevent bits of cotton adhering to them, since this would prevent a perfect seal.

After inoculation, the cotton stopper was discarded, and the rubber stopper carrying the tubes, was unwrapped and well flamed with a Bunsen flame. The rubber stopper was then pushed firmly into the neck of the bottle and covered with a thick layer of rubber cement. Thin blocks of wood were placed on the top of the stopper, and all wired in place with No. 16 iron wire.

After the bottle had been fitted, it was placed in a special incubator at 37°C. and immersed in a copper tank (M) containing heavy mineral oil. The lower end of the manometer tube was placed in a dish containing mercury, after removal of the cotton plug. The bottle was then exhausted as much as possible with a Geryk pump. When the mercury column ceased to rise in the manometer tube the tube attached to the pump was sealed at the constriction with a blast lamp. By this method we could obtain practically a complete vacuum as determined by the temperature and barometric pressure at the time. Figure 1 shows the details of this apparatus.

A series of cultures was prepared in exactly the same way and an analysis made at intervals as indicated in table 4.

#### METHODS OF ANALYSIS

*Gas analysis. (Total volume.)* The amount of gas could be calculated from the manometer readings and reduced to normal temperature and pressure by using the following formula:

$$V = \frac{273 V(P - T)}{760 (273 + t)}$$

in which  $V$  = volume,  $P$  = barometric pressure in mm. Hg.,  $T$  = vapor tension of water,<sup>2</sup> 760 = normal pressure, 273 = absolute temperature.

Determinations of the total volume of gas were recorded upon the bottle which remained in the incubator for the longest time. Manometer readings were taken on all the cultures at each interval but not all of these are included in the tables, since they would give no additional information as to the course of the fermentation.

*Method for collection and analysis of gas.* To obtain samples of gas, uncontaminated with air, the mercury pump was attached to the slender sealed tip (D) shown in figure 1. A tight fitting rubber stopper was first placed over the sealed tip and the rubber connection forced carefully over the tip until the metal tube came well down over the tip. This was wired firmly in place. Next the large rubber tube, which served as a receptacle for mercury, was drawn down and fitted over the stopper and wired in place. This large tube was filled with mercury to cover the connections completely.

The mercury pump was filled with mercury as completely as possible and the tube leading from the mercury pump to the connection with the culture container was exhausted with the Geryk pump. When the entire apparatus was exhausted, the connec-

<sup>2</sup> The vapor tension of water was used in all cases. This probably varied to some extent as the fermentation proceeded and new volatile products were formed. From the fact, however, that these were of unknown value, it was possible to use only the vapor tension of water in our calculations.

tion at D was given a slight bend to break the sealed tip. (If the tip has not been sealed too close to the lower part of the constriction it is very easily broken.) When the tip is broken there is an immediate fall of the mercury in the pump and a corresponding rise of the mercury column in the manometer tube. The gas is pumped out of the culture bottle and collected in a container over the mercury pump. The pumping is continued until the mercury column in the manometer tube becomes stationary.

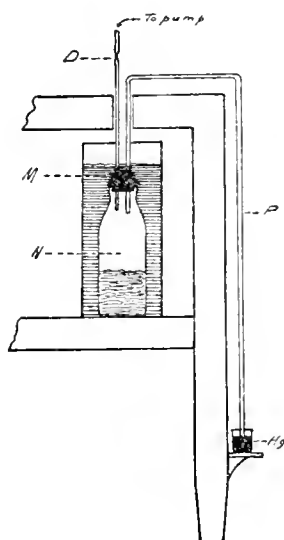


FIG. 1. SHOWING ARRANGEMENT OF CULTURES IN THE INCUBATOR



FIG. 2. SHOWING DETAILS OF THE CONNECTION BETWEEN THE CULTURE BOTTLE AND THE MERCURY PUMP

This is usually very close to the theoretical vacuum, and required about fifteen or twenty minutes. By this means it was possible to obtain the dissolved gases as well as those which had collected above the liquid. The liquid boiled vigorously at 37°C. and the water vapor probably aided in washing the gases out of the bottle.

After the gas had all been forced into the collecting bottle the connection between this bottle and the gas analysis apparatus

was opened and the mercury in the gas burette lowered. By this means a sample of gas was drawn in, measured and analysed. Figure 2 shows the details of the connection made at (D) figure 1.

The efficiency of our apparatus and pump was judged by the amount of oxygen in the gas obtained for analysis. In a few analyses we obtained a slight reduction in volume after absorbing the gas with alkaline pyrogallate solution. In such a case we calculated the air in the gas by multiplying the amount of decrease by five and making corrections in the calculation. These amounts were, however, usually nearly within the error in reading our burette. The apparatus used for the analysis of the gas is described by Burrell and Siebert (1913) and is a slight modification of that described by Haldane.

*Calculations.* The following example will show the calculations necessary in this connection. These were used throughout and for that reason the figures for each analysis are not included.

21.65 cc. of gas taken
5.63 cc. after CO <sub>2</sub> absorption
16.02 cc. of CO <sub>2</sub>
5.63 cc. after O <sub>2</sub> absorption
0.00 cc. of O <sub>2</sub>
18.45 cc. of CO <sub>2</sub> free air added
24.08 cc. total volume
16.85 cc. after combustion
7.23 cc. decrease due to combustion
7.19 cc. after CO <sub>2</sub> absorption
0.04 cc. of CO <sub>2</sub> due to combustion
4.82 cc. of hydrogen ( $\frac{3}{2}$ of decrease)
73.99 per cent CO <sub>2</sub>
22.26 per cent H <sub>2</sub>
3.71 per cent residual gas (by measurement)

The 0.04 cc. of CO<sub>2</sub> due to combustion was disregarded, since this amount is insignificant.

After the completion of the gas analysis, air was allowed to enter the apparatus through a cotton plug. The bottle was then removed from the incubator and the examination completed. The bottle was opened as carefully as possible and a sample taken for cultural purposes. From this sample stains, shakes and plates were made. The cultures were incubated several days at

37°C. From the shakes we could detect the presence of anaerobes, and plates showed the presence of aerobes. After we began the use of the present apparatus no contamination has been noted and we were always able to culture the anaerobe if growth had occurred.

Samples were also taken for the determination of ammonia, amino-acids and volatile fatty acids.

*Ammonia determinations.* The ammonia determinations were made by distilling in the presence of magnesium oxide. At first we tried the aeration method but even after eight hours we were able to obtain tests for ammonia with Nessler's reagent. After the gas analysis was made there was insufficient time for the aeration. It has been found in this laboratory, in working with fermenting mixtures of soil, etc., that there is a close correlation between duplicate samples run by the aeration and distillation methods. With small amounts of ammonia, the distillation methods, usually gave somewhat higher results than the aeration method; while with large amounts of ammonia present the results were reversed. When small amounts of ammonia (10 to 20 mgm. per 100 grams soil) were present the distillation method gave 3.6 per cent more ammonia than the aeration method. When large amounts of ammonia (60 to 75 mgm. per 100 grams soil) were present the aeration method gave 4.0 per cent more ammonia than the distillation method.

*Amino-acid determination.* Amino-acids were determined by the Van Slyke method. These determinations were complicated by the presence of very large amounts of ammonia. Van Slyke (1911) recommends the following method for the removal of ammonia: The ammonia can be removed by distillation with  $\text{Ca}(\text{OH})_2$  under diminished pressure, using a 10 per cent suspension until a slight excess is present, as shown by turbidity and alkaline reaction of the solution. The apparatus is evacuated to 30 mm. or less of mercury, and distillation continued for one-half hour at 45 to 50°C. Alcohol is added to prevent foaming.

We were unable to use this method because of lack of time and the difficulty of removing all the ammonia from the medium. As large amounts of ammonia were present and the temperature



of the laboratory was high (usually 25 to 35°C.), a correction for ammonia was necessary.

The following method was used in making these corrections. A 2 per cent peptone solution containing known amounts of standard ammonium hydroxide was placed in the Van Slyke apparatus and the amount of nitrogen determined in the usual manner. The difference between the peptone solution alone and a peptone solution plus ammonia was considered to be due entirely to ammonia.

Van Slyke states that about 15 per cent of the ammonia nitrogen present will be determined as amino-acid nitrogen at 20°C. in fifteen minutes. We obtained the following percentages by averaging the results obtained from duplicate determinations made on different dates.

Per cent of ammonia nitrogen that would be recorded as amino-acid nitrogen at different temperatures for the time used (five minutes).

At 21°C.	= 34.2 per cent
At 23°C.	= 39.6 per cent
At 25°C.	= 44.9 per cent
At 27°C.	= 50.6 per cent
At 29°C.	= 55.7 per cent
At 31°C.	= 61.1 per cent
At 33°C.	= 66.3 per cent
At 35°C.	= 71.6 per cent
At 37°C.	= 77.0 per cent

This means that the above per cents of the totalammonia nitrogen present would be recorded as amino-acid nitrogen at the various temperatures recorded.

It will be noted that these figures are considerably higher than those recorded by Van Slyke. The results obtained give a fairly uniform curve and were obtained under our experimental conditions. Of course, they were obtained by the use of a comparatively simple mixture, while in actual tests we were dealing with an extremely complex mixture. However, all the fermentation mixtures were different and it would have been impossible to simulate them closely.

TABLE 4  
Showing results of chemical examination

PRODUCTS FORMED	ACTION ON ASPARAGUS (100 GRAMS IN 300 CC. WATER)				ACTION ON ALKALINE EGG MEDIUM			
	Days incubated				Days incubated			
	2	4	6	8	2	5	8	12
Total cubic centimeters of gas...	148.0	251.0	257.0	280.0	66.0	77.0	102.0	112.0
Daily cubic centimeters of gas...	74.0	51.0	3.0	11.0	33.0	4.0	8.0	2.5
Per cent of CO <sub>2</sub> ...	98.6	97.2	97.1	95.3	64.9	71.3	70.1	73.7
Per cent of H <sub>2</sub> ...	0	0	0.5	3.3	26.4	17.8	23.9	16.8
Per cent residual gas.....	1.6	1.9	1.8	1.4	8.7	10.8	6.0	9.5
Cubic centimeters of CO <sub>2</sub> in gas...	146.0	244.0	250.0	267.0	43.0	55.0	72.0	83.0
Daily CO <sub>2</sub> in gas...	73.0	49.0	3.0	9.0	22.0	4.0	6.0	3.0
Cubic centimeters of H <sub>2</sub> in gas....	0	0	1.0	9.0	17.0	14.0	24.0	19.0
Daily H <sub>2</sub> in gas...	0	0	0.5	4.0	9	-1.0	3.0	-1.0
Ratio H <sub>2</sub> /CO <sub>2</sub> ....	-	-	1/250	1/29.0	1/2.4	1/4.0	1/3.0	1/4.4
Ammonia-N milligrams per 100 cc.	19.0	28.0	33.6	34.4	17.3	54.6	65.4	74.4
Daily ammonia-N milligrams per 100 cc.....	7.0	4.5	2.8	0.4	5.5	12.4	3.6	2.2
Amino-acid-N milligrams per 100 cc.....	27.83	35.14	61.86	69.20	67.56	103.35	93.08	87.75
Daily amino-acid-N milligrams per 100 cc.....	3.04	3.65	13.36	3.67	26.85	11.93	-3.42	-1.33
Cubic centimeters N/20 NaOH to neutralize volatile acids per 100 cc.....	9.10	11.70	11.00	8.20	-	6.80	9.60	15.60
Cubic centimeters of N/20 NaOH to neutralize volatile acids per 100 cc. daily. . .	3.05	1.30	-0.35	-1.40	-	2.26	0.93	1.50
10 cc.	6.5	5.1	5.4	7.3	-	6.30	8.8	11.5
20 cc.	13.1	10.2	10.8	13.4	-	10.5	14.7	20.5
30 cc.	19.7	15.4	15.3	19.5	-	14.6	19.6	26.9
40 cc.	26.1	22.5	21.6	27.4	-	17.7	24.6	33.3
50 cc.	32.5	27.9	27.0	32.3	-	21.7	28.1	38.4
100 cc.	48.3	44.4	45.1	51.2	-	39.6	48.5	52.5
200 cc.	68.1	66.6	65.1	73.1	-	60.4	62.0	65.3
300 cc.	79.0	78.6	78.2	85.2	-	72.5	76.7	71.8
400 cc.	85.6	85.4	79.6	90.1	-	80.8	85.6	76.9
500 cc.	89.9	90.5	81.4	92.5	-	87.6	88.5	80.7
	Before inoculation: Ammonia-N 5.0 mgm. per 100 cc. Amino-acid-N 20.75 mgm. per 100 cc. Volatile acids 3.0 cc. N/20 per 100 cc.				Before inoculation: Ammonia-N 6.2 mgm. per 100 cc. Amino-acid-N 13.85 mgm. per 100 cc. Volatile acids 0.0 cc. N/20 per 100 cc.			

TABLE 4—Continued

PRODUCTS FORMED	ACTION ON WHOLE MILK MEDIUM			ACTION ON 2 PER CENT PEPTONE WATER					
	Days incubated			Days incubated					
	5	8	12	3	5		11	14	
Total cubic centimeters of gas...	132.0	191.0	241.0	43.0	61.0	77.0	102.0	110.0	
Daily cubic centimeters of gas...	26.0	21.0	12.0	14.0	9.0	5.0	8.0	3.0	
Per cent of CO <sub>2</sub> ...	76.8	78.2	—	72.7	76.5	81.7	86.2	86.1	
Per cent of H <sub>2</sub> ...	15.0	17.5	—	17.3	15.2	12.6	5.2	8.9	
Per cent of residual gas.....	8.2	4.3	—	10.0	8.3	5.7	8.6	5.0	
Cubic centimeters of CO <sub>2</sub> in gas...	101.0	152.0	—	31.0	47.0	63.0	88.0	95.0	
Daily CO <sub>2</sub> in gas...	20.0	17.0	—	10.0	8.0	5.0	8.0	2.0	
Cubic centimeters of H <sub>2</sub> in gas....	20.0	31.0	—	7.0	9.0	10.0	5.0	10.0	
Daily H <sub>2</sub> in gas....	4.0	5.0	—	2.0	1.0	0.3	-1.6	1.6	
Ratio H <sub>2</sub> /CO <sub>2</sub> ....	1/5.1	1/1.5	—	1/4.3	1/5.3	1/6.3	1/17.6	1/5.9	
Ammonia-N milligrams per 100cc.	72.6	77.4	83.6	98.0	101.0	104.0	108.6	111.5	
Daily ammonia-N milligrams per 100 cc.....	12.7	1.6	1.5	32.0	1.3	1.0	1.3	0.9	
Amino-acid-N milligrams per 100 cc.....	83.14	92.45	124.47	132.98	112.04	103.74	33.48	32.56	
Daily amino-acid-N milligrams per 100 cc.....	11.80	3.11	7.99	19.59	-10.47	-2.77	-23.42	-0.64	
Cubic centimeters of N/20 NaOH to neutralize volatile acids per 100 cc.....	39.60	40.90	44.60	10.95	12.60	15.80	15.35	13.57	
Cubic centimeters of N/20 NaOH to neutralize volatile acids per 100 cc. daily....	6.91	0.43	0.92	3.20	0.55	1.06	-0.15	-0.59	
Per cent of volatile acids in fractions of distillate	10 cc.	8.5	8.9	8.9	7.20	6.6	5.8	6.3	7.2
	20 cc.	15.6	16.2	15.2	12.4	11.9	11.2	11.8	13.6
	30 cc.	21.7	22.6	21.9	19.1	16.7	16.3	16.5	19.6
	40 cc.	27.3	27.4	27.9	24.3	21.5	20.9	21.3	23.8
	50 cc.	32.8	31.4	32.8	29.6	26.3	25.6	25.6	27.9
	100 cc.	49.5	48.0	47.1	42.3	41.4	40.3	40.6	42.8
	200 cc.	66.1	65.6	61.0	60.4	58.2	58.1	59.5	61.1
300 cc.	74.2	74.9	67.8	72.2	69.7	68.2	69.4	71.2	
400 cc.	79.3	80.2	73.6	81.4	77.7	75.9	76.5	79.2	
500 cc.	82.6	84.6	77.6	81.6	83.4	81.8	81.6	84.8	
	Before inoculation: Ammonia-N 9.0 mgm. per 100 cc. Amino-acid-N 24.14 mgm. per 100 cc. Volatile acids 5.05 cc. N /20 per 100 cc.			Before inoculation: Ammonia-N 2.0 mgm. per 100 cc. Amino-acid-N 74.20 mgm. per 100 cc. Volatile acids 1.35 cc. N /20 per 100 cc.					

TABLE 4—Continued

PRODUCTS FORMED	ACTION ON 2 PER CENT PEPTONE SOLUTION, PLUS 0.5 PER CENT GLUCOSE			ACTION ON 2 PER CENT PEPTONE SOLUTION, PLUS 1 PER CENT LACTOSE			
	Days incubated			Days incubated			
	3	6	10	2	6	9	
Total cubic centimeters of gas...	208.0	312.0	333.0	82.0	128.0	133.0	
Daily cubic centimeters of gas...	69.0	31.0	5.0	41.0	12.0	2.0	
Per cent of CO <sub>2</sub> .....	62.2	68.4	90.0	75.9	84.5	88.2	
Per cent of H <sub>2</sub> .....	33.7	27.3	—	16.9	8.8	8.6	
Per cent residual gas.....	4.1	4.3	—	7.2	6.7	3.2	
Cubic centimeters of CO <sub>2</sub> in gas...	129.0	213.0	300.0	62.0	108.0	117.0	
Daily CO <sub>2</sub> in gas.....	43.0	28.0	22.0	31.0	15.0	3.0	
Cubic centimeters of H <sub>2</sub> in gas....	70.0	85.0	—	14.0	11.0	11.0	
Daily H <sub>2</sub> in gas.....	23.0	5.0	—	7.0	-1.0	0	
Ratio H <sub>2</sub> /CO <sub>2</sub> .....	1/1.8	1/2.5	—	1/1.5	1/9.8	1/10.6	
Ammonia-N milligrams per 100 cc.....	22.6	55.0	78.5	76.0	97.0	100.0	
Daily ammonia-N milligrams per 100 cc.....	6.8	10.8	5.9	37.1	5.2	1.0	
Amino-acid-N milligrams per 100 cc.....	79.81	97.95	113.12	109.76	116.14	65.40	
Daily amino-acid-N milligrams per 100 cc.....	7.57	6.04	3.79	21.68	1.59	-16.91	
Cubic centimeters of N/20 NaOH to neutralize volatile acids per 100 cc.....	24.2	32.5	59.8	12.75	11.85	10.30	
Cubic centimeters of N/20 NaOH to neutralize volatile acids per 100 cc. daily.....	7.6	2.8	6.8	6.10	-0.25	-0.52	
Per cent of volatile acids in fractions of distillate	10 cc.	2.5	4.9	6.0	5.3	6.8	5.9
	20 cc.	5.0	9.0	11.4	10.1	12.6	11.2
	30 cc.	6.7	14.8	16.4	14.9	17.9	16.2
	40 cc.	8.4	19.7	20.4	19.8	22.9	20.8
	50 cc.	10.3	23.8	24.1	23.7	27.6	24.7
	100 cc.	22.6	27.9	35.4	39.5	41.4	39.6
	200 cc.	42.7	41.9	54.5	58.4	62.5	51.1
	300 cc.	56.1	60.0	66.8	69.4	74.0	62.9
400 cc.	62.8	72.4	75.6	76.0	81.6	69.9	
500 cc.	67.7	80.6	81.2	80.8	86.9	75.0	
	Before inoculation: Ammonia-N 2.0 mgm. per 100 cc. Amino-acid-N 57.1 mgm. per 100 cc.			Before inoculation: Ammonia-N 1.8 mgm. per 100 cc. Amino-acid-N 66.39 mgm. per 100 cc. Volatile acids 0.55 per 100 cc.			
	Volatile acids 1.4 cc. N/20 per 100 cc.						

TABLE 4—Continued

PRODUCTS FORMED	ACTION ON 2 PERCENT PEPTONE SOLUTION, PLUS 1 PERCENT SUCROSE			ACTION ON 2 PERCENT PEPTONE SOLUTION, PLUS 1 PERCENT SALICIN			
	Days incubated			Days incubated			
	4	7	9	4	7	9	
Total cubic centimeters of gas....	5.0	28.0	42.0	43.0	208.0	228.0	
Daily cubic centimeters of gas....	1.0	8.0	7.0	11.0	55.0	10.0	
Per cent of CO <sub>2</sub> .....	—	18.3	52.4	—	61.9	65.8	
Per cent of H <sub>2</sub> .....	—	70.9	35.4	—	31.1	30.7	
Per cent of residual gas.....	—	10.8	12.2	—	4.0	3.5	
Cubic centimeters of CO <sub>2</sub> in gas..	—	5.0	22.0	—	129.0	150.0	
Daily CO <sub>2</sub> in gas.....	—	1.6	8.5	—	4.3	16.0	
Cubic centimeters of H <sub>2</sub> in gas....	—	19.9	14.9	—	71.0	70.0	
Daily H <sub>2</sub> in gas.....	—	6.6	—2.5	—	24.0	—0.3	
Ratio H <sub>2</sub> /CO <sub>2</sub> .....	—	4.0/1	1/1.5	—	1/1.8	1/2.1	
Ammonia-N milligrams per 100 cc.....	4.0	51.5	99.5	21.0	45.0	81.5	
Daily ammonia-N milligrams per 100 cc.....	0.5	15.8	24.0	4.8	8.0	18.3	
Amino-acid-N milligrams per 100 cc.....	71.81	77.46	83.79	61.81	77.46	83.79	
Daily amino-acid-N milligrams per 100 cc.....	—0.30	1.88	3.16	1.18	5.21	3.16	
Cubic centimeters of N/20 NaOH to neutralize volatile acids per 100 cc.....	3.00	17.30	13.40	5.90	20.20	42.60	
Cubic centimeters of N/20 NaOH to neutralize volatile acids per 100 cc. daily..	0.61	4.77	—1.95	1.34	4.77	11.20	
Per cent of volatile acids in fractions of distillate	10 cc.	5.0	6.9	6.8	1.7	5.5	5.8
	20 cc.	9.8	12.1	12.8	3.4	9.5	11.5
	30 cc.	13.3	16.8	18.4	5.1	13.5	16.4
	40 cc.	15.0	20.8	23.1	6.8	16.9	20.8
	50 cc.	16.7	24.2	27.3	7.6	19.4	24.8
	100 cc.	29.9	39.8	44.9	12.7	33.3	40.2
	200 cc.	46.6	57.7	63.7	19.5	56.6	59.3
	300 cc.	56.6	68.1	77.3	25.5	62.5	70.8
400 cc.	61.9	75.0	87.1	28.9	69.9	77.4	
500 cc.	71.6	80.0	94.8	32.3	75.3	82.5	
	Before inoculation: Ammonia-N 1.8 mgm. per 100 cc. Amino-acid-N 73.0 mgm. per 100 cc.			Before inoculation: Ammonia-N 1.8 mgm. per 100 cc. Amino-acid-N 57.08 mgm. per 100 cc. Volatile acids 0.55 cc. N/20 per 100 cc.			

TABLE 4—Continued

PRODUCTS FORMED	ACTION ON 2 PER CENT PEPTONE SOLUTION, PLUS 1 PER CENT MANNITOL			ACTION ON 2 PER CENT PEPTONE SOLUTION, PLUS 1 PER CENT GLYCEROL			
	Days incubated			Days incubated			
	2	6	9	4	7	9	
Total cubic centimeters of gas....	51.0	99.0	137.0	110.0	227.0	255.0	
Daily cubic centimeters of gas....	25.0	12.0	13.0	28.0	39.0	14.0	
Per cent of CO <sub>2</sub> .....	65.3	—	85.0	—	46.5	45.4	
Per cent of H <sub>2</sub> .....	27.2	—	10.8	—	44.5	4.8	
Per cent of residual gas.....	7.5	—	4.2	—	9.0	11.8	
Cubic centimeters of CO <sub>2</sub> in gas..	33.0	—	116.0	—	106.0	116.0	
Daily CO <sub>2</sub> in gas.....	16.0	—	27.0	—	35.0	5.0	
Cubic centimeters of H <sub>2</sub> in gas....	14.0	—	15.0	—	101.0	109.0	
Daily H <sub>2</sub> in gas.....	7.0	—	0.3	—	34.0	4.5	
Ratio H <sub>2</sub> /CO <sub>2</sub> .....	1/2.4	—	1/7.8	—	1/1.0	1/1.0	
Ammonia-N milligrams per 100 cc.	76.0	99.0	109.0	4.0	35.0	29.5	
Daily ammonia-N milligrams per 100 cc.....	37.1	7.7	3.3	0.55	10.4	-2.8	
Amino-acid-N milligrams per 100 cc.....	113.79	116.36	66.80	73.74	92.81	86.51	
Daily amino-acid-N milligrams per 100 cc.....	23.70	0.64	-16.51	0.18	6.35	-3.15	
Cubic centimeters of N/20 NaOH to neutralize volatile acids per 100 cc.....	22.45	27.50	32.90	4.00	9.40	10.50	
Cubic centimeters of N/20 NaOH to neutralize volatile acids per 100 cc. daily.....	10.90	-1.26	-1.80	0.86	1.80	0.55	
Per cent of volatile acids in fractions of distillate	10 cc.	6.7	5.2	4.6	2.5	5.3	6.9
	20 cc.	12.2	10.3	8.8	5.0	9.6	12.6
	30 cc.	16.9	15.1	12.8	6.3	12.8	17.4
	40 cc.	21.4	19.6	16.9	7.5	15.9	21.2
	50 cc.	25.4	23.6	19.1	17.5	19.3	25.0
	100 cc.	40.5	38.4	33.4	32.5	32.9	39.3
	200 cc.	58.8	57.3	52.9	45.4	58.0	55.5
	300 cc.	68.1	67.9	65.3	55.0	69.1	67.8
	400 cc.	74.8	75.1	73.5	62.5	76.6	74.8
500 cc.	80.1	81.3	79.6	67.5	81.9	79.5	
	Before inoculation: Ammonia-N 1.8 mgm. per 100 cc. Amino-acid-N 66.39 mgm. per 100 cc. Volatile acids 0.65 cc. N/20 per 100 cc.			Before inoculation: Ammonia-N 1.8 mgm. per 100 cc. Amino-acid-N 73.02 mgm. per 100 cc. Volatile acids 0.55 cc. N/20 per 100 cc.			

TABLE 4—*Continued*

PRODUCTS FORMED	ACTION ON 2 PER CENT PEPTONE SOLUTION, PLUS 1 PER CENT SOLUBLE STARCH				ACTION ON 2 PER CENT PEPTONE SOLUTION, PLUS 1 PER CENT INULIN			
	DAYS INCUBATED				DAYS INCUBATED			
	4	6	9	12	4	6	10	
Total cubic centimeters of gas.....	18.0	113.0	213.0	316.0	28.0	30.0	33.0	
Daily cubic centimeters of gas.....	4.0	47.0	33.0	31.0	7.0	1.0	1.0	
Per cent of CO <sub>2</sub> .....	44.1	73.6	71.2	79.3	20.6	29.3	42.4	
Per cent of H <sub>2</sub> .....	53.6	23.5	21.7	18.0	—	70.0	50.6	
Per cent of residual gas..	2.3	2.9	4.1	2.7	—	0.7	7.0	
Cubic centimeters of CO <sub>2</sub> in gas.....	8.0	83.0	151.0	251.0	6.0	9.0	11.0	
Daily CO <sub>2</sub> in gas.....	2.0	38.0	23.0	33.0	1.5	1.5	1.5	
Cubic centimeters of H <sub>2</sub> in gas.....	10.0	27.0	53.0	57.0	—	21.0	17.0	
Daily H <sub>2</sub> in gas.....	2.5	8.0	5.0	1.0	—	10.0	-1.0	
Ratio H <sub>2</sub> CO <sub>2</sub> .....	1.2.1	1.3.1	1.2.9	1.1.1	—	2.4.1	1.2.1	
Ammonia-N milligrams per 100 cc.....	40.0	50.0	52.5	81.0	47.5	60.0	69.0	
Daily ammonia-N milligrams per 100 cc.....	9.5	5.0	0.8	9.5	11.4	6.3	2.2	
Amino-acids-N milligrams per 100 cc.....	67.26	82.50	89.90	108.51	61.52	64.20	82.54	
Daily amino-acid-N milligrams per 100 cc.....	0.68	7.62	2.43	6.20	-0.75	-1.34	4.58	
Cubic centimeters of N/20 to neutralize volatile acids per 100 cc.....	3.50	16.15	23.95	31.65	1.10	—	11.9	
Cubic centimeters of N/20 to neutralize volatile acids per 100 cc. daily..	0.70	6.33	2.60	2.58	0.10	—	2.7	
Per cent of volatile acids in fractions of distillate	10 cc.	17.1	11.1	20.8	23.6	4.5	—	6.7
	20 cc.	31.2	20.8	39.9	41.8	9.1	—	13.4
	30 cc.	42.8	28.9	50.1	53.9	13.6	—	16.3
	40 cc.	54.3	35.4	58.2	60.0	22.7	—	24.4
	50 cc.	62.8	41.9	64.2	66.1	50.0	—	29.4
	100 cc.	88.5	63.0	90.2	96.4	68.2	—	51.2
	200 cc.	97.1	90.1	98.1	99.4	77.2	—	73.1
300 cc.	99.9	97.1	—	—	81.8	—	88.2	
400 cc.	—	—	—	—	—	—	94.1	
500 cc.	—	—	—	—	—	—	97.4	
	Before inoculation: Ammonia-N 2.0 mgm. per 100 cc. Amino-acid-N 64.54 mgm. per 100 cc.  Volatile acids 0.70 cc. N/20 per 100 cc.				Before inoculation: Ammonia-N 2.0 mgm. per 100 cc. Amino-acid-N 64.54 mgm. per 100 cc.  Volatile acids 0.70 cc. N/20 per 100 cc.			

Since there were such large amounts of ammonia present, and because it is so difficult to remove it from the sample, corrections by the use of the factors have probably given results as nearly correct as could have been obtained by any other method.

*Volatile fatty-acid determinations.* For the determination of volatile fatty-acids we have followed the Duclaux method as modified and described by Dyer (1916). This author suggested that the method might be employed in the study of acid production by bacteria, and Wolf and his associates used it for the study of the acid production by certain pathogenic anaerobes.

While the results which we have obtained show that there is a difference in the fermentative activities of this organism upon the different carbohydrates the differences are not great. The distillation of volatile fatty acids is not an easy task and while its use may be of value in detecting in a general way what products are formed during a given fermentation, their value as a diagnostic biochemical test for bacterial differentiation is limited.

Our attitude toward this test cannot be better expressed than by quoting from the summary of Wolf and Telfer.

An experimental critique of the Dyer method of estimating volatile fatty acids has been made. This method, while perfectly satisfactory in the form stated by its author in dealing with a mixture of two volatile acids, the nature of which is known, fails when a mixture of unknown acids is to be analyzed. The color tests as proposed by him are satisfactory when dealing with pure acids, but not as positive as could be desired for the identification of an acid in the mixture. The separation of the acids is necessary before any reliance can be placed on these color tests.

From a study of table 4, it may be seen that in most cases the fractionation curves lie very near to those of propionic and butyric acids. By the tests devised by Dyer we could obtain no evidence of propionic acid, though various fractions were refractionated as indicated by Wolf and Telfer. We could obtain some fairly good tests for acetic and butyric acids after repeated refractionations; in the case of milk, the odor would indicate valerianic acid though no qualitative test could be obtained for this or for caproic acid.



The results indicated that the curve was due not to propionic acid but rather to a mixture of acetic, butyric and perhaps to valerianic acid. We felt, however, that work of this sort led to rather uncertain results, since we were able to simulate almost any acid curve desired by mixtures of other acids in the proper proportions.

The refractionation method proposed by Wolf and Telfer was not exhaustive enough to obtain various acids in a form sufficiently pure for accurate tests.

The fractional distillation of the cultures was completed in an attempt to find differences wide enough to be of diagnostic value, even though the acids present were not completely identified.

#### DISCUSSION OF RESULTS

An examination of table 4 will give an idea of the action of these organisms upon media rich, both in protein and carbohydrates. The cultures were very closely related in all respects in their biochemical reactions.

In most respects the quantitative results compare favorably with the qualitative results, but unfortunately do not give much additional information. Those showing large amounts of gas and acid in fermentation tubes, and Hiss serum water media containing the various carbohydrates and grown over phosphorus in an anaerobic jar, also show quantitatively larger amounts of gas and acid when grown in vacuum. It was impossible to obtain duplicates which checked exactly. Variation is inherent in all bacteriological work and the results obtained in this work check about as closely as those in many other bacteriological determinations.

*Probable error.* Two series of cultures were set up to determine the probable error. One series of five bottles containing two percent peptone-water after four days contained the following amounts of gas 92, 86, 61, 73, and 87 cc., respectively. A series of six bottles containing whole milk after two days contained the following amounts of gas 237, 243, 232, 203, 196, 186 cc., respectively. By using the formula:

$$E = 0.6745 \sqrt{\frac{S}{N(N-1)}}$$

in which  $E$  = probable error,  $S$  = sum of residuals,  $N$  = number of parallel determinations. The probable error as determined for the first series is  $\pm 4.78$ , and for the second series is  $\pm 3.07$ .

The probable error was not determined for ammonia, amino-acid, or volatile-acid production, but duplicate determinations usually varied about as much as in the gas determinations.

*Nature of fermentation.* It will be noted that this organism produced considerable amounts of gas from peptone solution. This probably accounted for the presence of bubbles of gas in fermentation tubes containing non-fermentable carbohydrates. The higher negative tension and the larger amounts of media in the bottles tended to magnify the gas production from this medium in bottles, as compared to that in tubes in the anaerobic jar. (The latter usually showed a tension of about 350 mm. of mercury.)

When we mention the fact that an organism ferments a certain carbohydrate we usually refer to the fact that the change results in the production of gas or acid. These are products easily determined and are evidently due to deep-seated changes in the product. It is quite probable, however, that there may be organisms which bring about but slight changes in a carbohydrate, not extensive enough to lead to the formation of acid or even gas. It is also possible that these products may be produced and again utilized in the synthesis of cell protoplasm, or changed to other products of fermentation. It is a well known fact that the nitrifying bacteria utilize  $\text{CO}_2$  as a source of carbon in the oxidation of ammonia and nitrites to nitrates. Nikitinsky (1907) showed that there were certain anaerobes which were able to utilize hydrogen. For this purpose he used "konzentrierten kanalisationsflüssigkeit" and "Schlamm aus einem Absitzbecken" placed in an atmosphere of hydrogen. In one case he found that 500 grams of "Schlamm" was able to combine with an average of 30 cc. or a maximum of 70 cc. of hydrogen per day.

It is a well known fact that many bacteria can utilize acids and alcohols as a source of energy. It may also be possible that bacteria utilize only a minimum amount of the carbohydrate, enough to establish their initial development, but do not produce

changes, deep-seated enough to be detected by the ordinary chemical methods. After growth has been established they may then utilize some other element in the medium as their chief source of energy. It may be noted that the anaerobes studied by us grow very well in a solution of peptone and water.

These organisms could not utilize simple nitrogenous foods to any extent; nitrates, ammonia salts, urea and asparagin in a simple solution or in the presence of glucose give practically no development. They were able to grow well upon simple peptone solutions and attack the albumins of egg-white, blood serum, milk and meat, and also liquefy gelatin, either in the presence or absence of glucose. The changes in all cases are deep-seated since gas, volatile fatty acids, amino-acids and ammonia are formed from all of these media in fairly large amounts. Traces of hydrogen sulphide are also found in some of the meat cultures.

The proteolytic action is tryptic in nature, since it does not take place in a medium acid to litmus and is more active in a medium neutral to phenolphthaline than in one neutral to litmus.

*Gas production.* In cases in which large volumes of gas were present we considered that active fermentation had occurred, and this was correlated with the results of the fermentation tube tests. From these results, we are safe in considering that an anaerobe has attacked a carbohydrate vigorously only when it produces more than 1 per cent of gas in the closed arm of the fermentation tube, and a reaction distinctly acid to brom-thymol-blue. A bubble of gas might be taken to indicate slight fermentation of the carbohydrate, if it were present in the tube containing carbohydrate medium but not in a tube containing the media minus the carbohydrate. We have made as many as twenty parallel cultures of these anaerobes and treated all exactly alike; in some tubes there may be as much as one per cent of gas, in others a small bubble, and in others none at all, and all containing about an equal amount of growth. Since irregularities are unavoidable with these organisms, some arbitrary limit should be set. In this work we recorded a positive fermentation when more than one per cent of gas was formed and a questionable result when less than that amount was present if there were no marked change in the reaction.

In the case of lactose, inulin, arabinose and xylose there appears to be no doubt that these carbohydrates were not attacked to any extent. With mannitol there was an increase of gas and but little increase in acid above that in peptone solution alone. We have concluded that there was a slight fermentation of mannitol.

In most experiments the predominating gas was  $\text{CO}_2$ . Hydrogen was obtained in all cases except with the cultures upon asparagus at the beginning of the fermentation. One point of interest in this connection is the ratio of  $\text{H}_2$  to  $\text{CO}_2$ . In all our tests the amount of  $\text{CO}_2$  increased with the age of the fermentation, while the  $\text{H}_2$  remained practically stationary after the first few days. This caused a change in the ratio of  $\text{H}_2$  to  $\text{CO}_2$  from day to day. Most authors mention a ratio between these gases as determined at a certain time. By determining it at intervals the ratio might be somewhat different as shown in this case. Apparently the ability of the organism to form hydrogen was limited to the beginning of fermentation; or the substance from which this gas was formed was used up in the earlier stages; or the hydrogen was oxidized as it was formed, as has been suggested by Nikitinsky, (1907) and Kaserer (1906).

According to Perdrix (1891) *B. amylomyze* produced a gas of variable composition with the different carbohydrates at different ages of the culture. He gives the following table to show this (for glucose).

TIME	V		R	D	
	Hydrogen	Ac. carb.		Hydrogen	Ac. carb.
<i>days</i>					
3	175	85	2.0	175	85
4	275	145	1.9	100	60
5	350	220	1.6	75	75
11	670	450	1.5	120	130

V = volume, R = ratio, D = difference.

Grimbert (1893) found that his *B. orthobutylicus* growing in glucose solution without chalk produced the following results:

	HYDROGEN	AC. CARB.	R
Up to and including 4th day.....	11.66	10.00	1.16
4th to 13th day.....	11.24	32.76	0.34
13th to 22nd day.....	1.90	6.90	0.28
	24.80	49.66	0.50

In our work the irregularities in the total amounts of the various gases were due to the fact that the amounts of gas were determined from the bottles which remained in the incubator for the longest time, and only part of these were analyzed at each period. An examination of the gas volumes as mentioned on page 398 will explain this variation.

In all cases there was considerable residual gas which could not be accounted for by the amount of air remaining in the bottles, since these were exhausted to nearly the theoretical vacuum. No doubt this was nitrogen gas which had been liberated from the medium. In no case was there more than a trace of  $\text{CO}_2$  formed by combustion with oxygen. For this reason we may say that no methane or ethane were formed by these organisms.

It will be noticed from table 4, that gas is produced slowly in all cases except in presence of glucose and glycerol. These substances are easily fermented and all cultures produce gas in large amounts. In the presence of lactose, there is about the same action as upon peptone solution except that slightly more hydrogen is produced in some cases, and the growth is slightly more vigorous. A slight action upon the lactose may have occurred in the latter case.

*Ammonia.* The ammonia production is not always in inverse ratio to the production of gas and acid by these organisms. The cultures showing larger amounts of gas show smaller amounts of ammonia in some cases but not in all. Glucose reduced the ammonia production for this organism. In the presence of lactose, mannitol and sucrose there was practically no reduction in the ammonia formation. In the presence of salicin there was a decrease, and the organism is to be considered a salicin fermenting type. Peptone solution plus glycerol shows much less ammonia

produced and there was clear evidence that the organisms attacked the glycerol. There seemed to be vigorous growth in inulin-peptone solution but the action upon the medium was not so vigorous.

The proteolytic action of these organisms is also shown by the production of ammonia from asparagus, alkaline egg medium and whole milk.

*Amino-acids.* In the liberation of amino-acids from the peptone solution plus carbohydrates there is a correlation similar to that seen in the production of ammonia from similar solutions. From peptone alone there is a marked decrease in the amino-acid content after the first three days.

The freeing of amino-acids does not correspond to the case with which a carbohydrate is fermented. There is an increase in the amino-acid content for a certain period in all cultures. In the presence of glucose, salicin, sucrose, starch and inulin this increase continues to the close of the experiment. With lactose and glycerol there is a slight decrease as the culture ages. In case of mannitol there is a decrease from the beginning. There is a marked production of amino acids from asparagus, alkaline egg medium, and whole milk. In a purely nitrogenous media there is a marked increase in the amino-acid nitrogen, followed by a decrease as seen in alkaline egg and peptone solutions. This point is not so marked in media containing fermentable compounds such as asparagus, milk, glucose, salicine, glycerol and starch solutions. Apparently the organisms may not attack the amino-acids as readily in the presence of fermentable carbohydrates as they do in their absence, or they may be produced more rapidly than they are used and thus accumulate in the medium. It is impossible to elucidate this point at present since total nitrogen determinations were not made on each sample at various periods.

*Volatile fatty acids.* Fairly large amounts of volatile fatty acids were produced in most cases. The figures in table 4 represent cubic centimeters of N/20 alkali used to neutralize the acid obtained by complete distillation, as above described. The per cent of volatile acid in each fraction was obtained by dividing the amount by the total acid. Usually there was a change in the

total amount of volatile acid, and in most instances some change in the fractionation constant from day to day. With peptone alone, there was little change, but with glucose, salicin, glycerol, and soluble starch, there was an increase, while mannitol caused a decrease. These differences were constant and are probably due to a slight difference in the fermentation reaction which occurred in these cultures at different ages. The increase in the amount of acid obtained in the first fractions would indicate an increase in the higher acids; a decrease in the amount of acid obtained in the first fractions would indicate an increase in the lower acids over those originally present.

Less acid was produced in the presence of glycerol and soluble starch than was produced from peptone alone. Apparently the gas and acid production are not always related in the fermentation of the various carbohydrates. This is shown to be especially true of glycerol and soluble starch in which there are very large amounts of gas and only small amounts of acid formed. The distillation curves for glucose, glycerol and inulin indicate the presence of an acid of low molecular weight, probably acetic acid; while curves obtained from starch indicate large amounts of acids of high molecular weight, probably butyric. Usually the same type of curve was obtained throughout the entire incubation period, indicating that the difference in the gas ratio is not closely correlated to the production of different kinds of acid. Apparently the  $\text{CO}_2$  in the later stages of fermentation comes from the amino-acids present and not from the carbohydrates.

#### CONCLUSIONS

1. We have described a method adapted for the quantitative study of the decomposition products formed by anaerobic bacteria.
2. The method described has been found very satisfactory for the cultivation of anaerobic bacteria. It has also proven very satisfactory as a means of measuring the amount and kind of gas produced.
3. The objection to the use of such an apparatus is that it does not give the anaerobes an oxygen tension which might be more favorable to their development. Even the "obligate" anaerobes

probably utilize a certain amount of free oxygen when it is available.

4. The quantitative results obtained in this particular case do not happen to yield results of significance in the subdivision of the group of organisms studied. They do, however, give us a clear picture of the biochemical behavior of these types which will make possible an accurate comparison of their physiology with that of other forms which may later be investigated.

5. It may be hoped that chemists will interest themselves in the improvement and simplification of chemical methods necessary to determine quantitatively and biochemically changes due to the growth of microorganisms in various culture media. As such methods are devised the bacteriologist will obtain an increasingly sound and logical basis for the differentiation of microbial species, and will obtain much valuable information concerning the nature of the life processes of microorganisms.

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THE PROPORTION OF VIABLE BACTERIA IN YOUNG  
CULTURES WITH ESPECIAL REFERENCE  
TO THE TECHNIQUE EMPLOYED  
IN COUNTING

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PART I

During the course of some work on the morphological changes occurring during the life history of certain bacteria, it became desirable to ascertain the true relationship existing between the number of living organisms and the total number of cells in a young broth culture. A few preliminary experiments were sufficient to show that a certain discrepancy existed between these numbers in spite of the fact that it has been frequently stated, and in many cases taken for granted, that in a young broth culture—up to twenty-four hours—all the bacilli are viable. On looking up the literature it was found that of the many observers who had made a comparison of the two counts—Hehewerth (1901), Zelikow (1906), Eijkman (1904), Winterberg (1898), Klein (1902), Wright (1902), Anderson, Fred and Peterson (1920) and Glynn, Powell, Rees and Cox (1913)—not one had succeeded in proving the two to be identical. In fact, in the majority of cases, a definite numerical inferiority was found in the case of the viable count. In nearly every case this discrepancy was passed over with but little comment, the results being explained by errors in the technical procedure adopted. The argument appears to have been that because in the early stage of a bacterial culture all the bacilli are living, then any failure in the agreement of the total and viable counts must be due to technical error. To determine

this point exactly it was clear that a technique would have to be evolved which would permit of as small an error as possible.

A careful study of the literature seemed to show that few of the methods which have been employed hitherto could claim to conform to such a degree of accuracy. Generally speaking, the methods which have been employed may be classified into (1) the direct and (2) the indirect. In the former the organisms are counted directly under the microscope, in the latter the number of bacteria present is calculated from an enumeration of the colonies which develop when an aliquot part of the emulsion in question is mixed with a nutrient medium in a Petri dish, and incubated for a variable period of time. The former is designed to record the total number of organisms present, the latter only the number which happens to be viable at the moment of sampling.

With regard to the direct or total count, Klein (1900) appears to have been one of the first to realize the value of such a method of estimation. His technique consisted in staining a moist preparation of organisms with aniline gentian violet, spreading a loopful of known capacity on a coverslip, drying, and clearing in xylol balsam. The total number of fields on the coverslip was determined for a definite combination of lenses, fifty fields were counted and the number of stainable organisms per cubic centimeter in the original culture was calculated. This technique was followed by Hehewerth and Eijkman, and in a somewhat modified form by Anderson, Fred and Peterson. Zelikow introduced a method whereby the number of bacteria was determined by estimating the amount of dye they were able to adsorb from a solution of fuchsin, the strength of the latter before and after adsorption being determined by means of a Duboseq colorimeter. Winterberg conducted his count in a Thoma-Zeiss chamber. Wright's method of counting against red blood cells is too well known to need description. A modification of it was described by Harrison (1905) who observed the mixture of bacteria and red cells in a moist film instead of in the dried condition. Finally, a new form of counting chamber—the Helbe—similar to the Thoma-Zeiss, but measuring only 0.02 mm., in depth, and fitted with an opti-

cally plane cemented coverslip for use with a  $\frac{1}{12}$  inch objective, was employed by Glynn, Powell, Rees and Cox, the organisms being examined in the stained condition under open illumination.

The indirect or viable count has, as a rule, been performed by a modification of Koch's original plating method. The only exceptions which need be noted are those of Naegeli and Schwendener (1877) who took the amount of fermentation as their basis for the computation of the number of growing organisms, and Winterberg who accepted motility as his criterion of viability. The modifications of Koch's method have been concerned with the medium used, the question of preliminary dilution, the methods of dilution and the exact technique of counting the plates. The majority of observers appear to have used agar, but Buchner, Longard and Riedlin (1887), Hehewerth, Eijkman, Zelikow, and Chick (1912), seem to have preferred gelatin, though in some cases both media were employed. With regard to preliminary dilution, the earlier workers generally preferred to plate out the original emulsion, while of late the tendency has been in the opposite direction, as in the case of Winterberg, Zelikow, Müller (1895), Rahn (1906), Madsen and Nyman (1907), Lane Claypon (1909), Penfold (1914), Coplans (1909), Chesney (1916), and Noyes and Voigt (1916). The method of dilution has been subject to considerable variation; on the whole volumetric pipettes have been most popular, but Lane Claypon, Ficker (1898), Chick, and Penfold used dropping pipettes, while Hehewerth, and Graham Smith (1920) resorted to the use of a standard platinum loop. Gotschlich and Weigang (1895) elaborated a special technique in which dilution was performed by a combination of gravimetric and volumetric methods. The important question of the counting of the plates has naturally depended largely on whether or no a preliminary dilution of the emulsion was made. Where the number of colonies was very great, microscopic counting was adopted, usually with the aid of a Wolfhügel's plate, as in the case of Buchner, Longard and Riedlin, Where on the contrary, dilution was employed, the use of the microscope was no longer necessary, and counting was performed with the naked eye or with a magnifying glass—Krönig and Paul,

Zelikow, Neisser, Lane Claypon, Penfold, and Moore (1915). Graham Smith used a dissecting microscope, while Frost (1921) has evolved a microplate method for purposes of rapid enumeration of organisms in milk. The foregoing summary of the literature is only intended to indicate the main outlines which have been followed, a complete survey lying beyond the scope of this paper.

In perusing the results of previous workers, it was striking to observe the peculiar lack of attention which was paid to the estimation of the experimental error involved in the methods employed. Probably this is to be attributed to the fact that in many cases in which the enumeration of bacteria was undertaken, a relative, rather than an absolute accuracy was essential. It was felt that the successful accomplishment of this object could only be attained by working out a technique in which the errors inherent in every step should be known with certainty. The fulfillment of this requirement has been kept constantly in mind throughout the course of the present work.

#### *Technique of the total count*

After careful consideration and personal experience of several methods—especially those of Wright and of Brown and Kirwan (1915)—it was decided to adopt the method which has been in use in this laboratory during the past two years. Practically, this resolves itself into the adaptation of the Helbe counting chamber to dark-ground illumination. Instead of examining the organisms in a stained condition with open illumination they are observed in their natural state against a dark background. For use with a paraboloid condenser, the only alteration of the chamber which is required, is a selection of a slide of such a thickness that the distance between the lower surface of the chamber and the upper surface of the condenser shall lie between 0.9 and 1.1 mm. With a slide of other dimensions than these the correct focussing of the rays of light becomes impracticable. The depth of the chamber is 0.02 mm., while the surface is ruled into small squares.<sup>1</sup> The best combination of lenses has been found to be

<sup>1</sup> A slide of the dimensions quoted was made by Messrs. Hawksley & Son.

a two-thirds objective and an eighteen compensating ocular. Preliminary dilution of the emulsion is made—where necessary—by means of dropping pipettes—to be afterwards described—the diluent used being a 1 per cent solution of phenol in 0.9 per cent saline. If the preparation is examined immediately after adjustment of the coverslip, the bacilli stand out as light, refractile rods having a yellowish color, and showing a certain amount of Brownian movement; after a lapse of ten or fifteen minutes, the bacilli begin to settle on the floor of the chamber and of course lose their molecular motion; though in this condition they are quite easy to recognize, it is better to examine the preparation directly after it is put up so as to have the advantage of the Brownian movement, the degree of which was soon recognized to be characteristic of bacilli, as opposed to that possessed by minute particles of albumins and other material which is of a far more active nature.

The only serious drawback that has been encountered in the use of this method is that it is unsuitable for dealing with broth emulsions containing fewer than fifty million organisms per cubic centimeter. This objection is generally of little importance, for the majority of counts one desires to make are concerned with much thicker emulsions, but the force of the disadvantage became clear when we wished to perform total counts on the early stages of a young broth culture before any visible turbidity had appeared. For this purpose we at first resorted to the use of a Thoma-Zeiss slide of 0.01 mm. depth, the bacilli being suspended in a weak solution of methylene blue, and examined by open illumination. Experience showed, however, that such a method was unreliable, errors of 50 per cent in comparative counts being met with; it was therefore abandoned altogether. So far, no satisfactory solution of this difficulty has been found.

#### *Estimate of the error of the total count*

Throughout most of the work to be described in this paper, a particular strain of *Bact. suispestifer* has been used, but for some of the earlier counts the stock strain of *Bact. typhosum* was selected.

In order to ascertain the error involved in the actual process of counting, three experiments were performed on three separate occasions. On the first occasion an emulsion of *Bact. suipestifer* in 1 per cent phenolised saline was counted eight times in succession, and the results compared. They are shown in table 1, the counts being in terms of the original emulsion.

On the second occasion, using an emulsion of *Bact. typhosum*, five counts were made, with a resulting percentage mean error of 4.04 per cent. On the third occasion ten counts were performed with a resulting percentage mean error of 4.95 per cent.

TABLE 1

NUMBER OF COUNT	COUNT PER CUBIC CENTIMETER
1	13,830,000,000
2	14,400,000,000
3	15,130,000,000
4	14,730,000,000
5	15,830,000,000
6	14,100,000,000
7	15,000,000,000
8	14,830,000,000
Arithmetic mean.....	14,730,000,000
Mean error.....	464,000,000
Percentage mean error.....	3.15

From these three experiments it is seen that the mean error of performing the total count under the conditions described may be taken to be not greater than 5 per cent. It is interesting to compare these results with those obtained by Glynn, Powell, Rees and Cox. Working with staphylococci, streptococci, and *Bact. coli* they found that in the case of an 0.02 mm., chamber the average percentage deviation from the arithmetic mean was 3.1 per cent; with an 0.1 mm., chamber it was 5.4 per cent, and with Wright's blood film method it was 34.1 per cent. With regard to Klein's method, Gotschlich (1912) quotes the mean error of the total count as 19 per cent.



*The method of dilution*

Before proceeding further it will be necessary to give details of the methods of dilution which have been employed in both the total and the viable counts. For this purpose dropping pipettes have been preferred to volumetric pipettes, chiefly because in following out the growth of a bacterial culture such a large number of volumetric pipettes are required that the price of the latter becomes prohibitive: moreover dropping pipettes are more convenient to handle and more accurate in their delivery.

For full details with regard to the preparation of these pipettes and for an account of the precautions to be observed in handling them, the original article by Donald (1915) should be consulted. Suffice it to say that his technique has been used throughout with the exception of one or two modifications. Thus, for the purpose of calibration, an Imperial Standard wire gauge has been employed and, in order to insure accuracy, the pipettes have been subsequently tested by means of an ordinary screw micrometer measuring to 0.01 mm. Further, the drop values have been estimated by gravimetric, instead of by volumetric methods. The actual size selected was no. 22 on the wire gauge. After working out the drop volumes of various fluids at the temperature at which they were to be used, one factor remained to be determined, namely the effect of variations in density of a bacterial emulsion on the size of the drop delivered. On investigating this point, it was found, contrary to expectation, that there was no appreciable difference in the drop values of emulsions ranging in thickness from 500,000,000 to 10,000,000,000 organisms per cubic centimeter. The explanation of this would appear to be that as the emulsion increases in density, the weight of the organisms in the drop just suffices to counterbalance the increasing viscosity of the liquid, so that the actual value of the drop remains approximately constant.

*Errors of the dropping pipettes*

In order to determine the errors of the dropping pipette a considerable number of experiments were made, the details of which

it is not proposed to enter into here as the space required would be greater than would be warranted by their relative importance. The results arrived at may be set out in the following order: (1) In making any dilution not less than four drops should be used, as with a smaller number sufficient accuracy is difficult to obtain. With four drops, however, or more, the error of delivery does not exceed 1.2 per cent. (2) The bacterial content of drops delivered in succession appears to be uniform; no difference between individual drops could be substantiated. (3) So long as the interior of the pipettes is clean—and all pipettes should be washed through with alcohol and ether—only a comparatively small number of bacteria remains adherent to the walls; this error of clinging does not appear to be greater than 1 per cent. (4) In making serial dilutions a separate pipette should be used for each emulsion. Under these circumstances the deviation of the actual from the calculated dilution was found to be not greater than 3 per cent.

*The employment of roll tubes for the estimation of the viable count*

Before entering on a complete description of the method employed in the estimation of the viable count, it is thought advisable to give a brief resumé of some of the technical details which had to be worked out in order to insure the greatest degree of accuracy. In the first place Petri dishes have been discarded in favor of roll tubes. These are prepared in the following manner: Test tubes measuring 6 inches by  $\frac{5}{8}$  inch are selected; after sterilization in the hot oven, about 2 cc., of nutrient agar is placed in each. They are then autoclaved for twenty minutes at 120°C. When required for use the agar is melted and allowed to cool to 45°C., in a water bath; the inoculum is delivered directly into the tube, the contents of which are mixed by gentle shaking. The tube is then rolled between the fingers—as in the case of the Esmarch roll tube—the agar being allowed to pass about halfway up the tube. As a rule solidification is complete in half to one minute, and the tubes are then incubated in an inverted position for three days at 37°C. At the end of that time, when they are removed, it will be found that the agar is studded with colonies varying from round to lenticular in shape, evenly distributed

throughout the tube. In order to count the number which has developed, a series of circles, 1 cm. apart, is lightly drawn around the tube with an oil-marking pencil, and a longitudinal line drawn from top to bottom intersecting each circle at right angles. The actual counting is performed by means of a small magnifying glass, the tube being examined against a dark back-ground illuminated by a partially concealed electric bulb. The contents of each circle are observed in turn from above downwards, till the total number of colonies in the tube has been enumerated. This procedure is greatly facilitated by the registration of each colony on a small counting machine—a process which has the double advantage of not only saving one the trouble of memorizing the figures, but also of eliminating the personal factor in counting, for one has no idea of the actual number till the tube has been completely examined.

In using roll tubes for this purpose one or two small points should be noticed. The temperature of the agar at the time of inoculation may be allowed to vary between 41° and 50°C.; no deleterious effect of a temperature even as high as 55°C. could be noticed in experiments made to determine this point. Further, it is well to allow the agar tubes after melting to remain in a water bath at 45°C. for five to ten minutes, so as to allow of the condensation of some of the excess moisture suspended in the air of the tube, otherwise it may be deposited on the surface of the agar after rolling, and lead to an undesirable amount of spreading. It is to allow any such moisture to run down on to the cotton plug and so be absorbed that the tubes are incubated in an inverted position. Finally to prevent excessive condensation, it is advisable to place the tubes in the incubator as soon after rolling as possible.

#### *Comparison of tubes and plates*

The question will naturally arise as to the reason for the abandonment of plates in favor of tubes. In reply the following factors may be adduced: (1) The prohibitive cost of plates when large numbers are required for use. In many experiments 40 and 50 tubes have been used at a time, and as these experiments

have been repeated from day to day, the acquirement of a stock of 200 or 300 plates would have been necessitated, had these been employed. (2) There is considerably less risk of contamination in the case of tubes. Absolute sterility is obtained by autoclaving the agar. Contamination, in fact, does not occur. In dealing with several thousand tubes during the course of this work, only one single case of contamination has been encountered. With plates, on the contrary, the risk of contamination is by no means negligible, especially if incubation has to be continued for three days. (3) Less media is required in the case of tubes than with plates; for the former 2 cc. are sufficient, for the latter—if a 5-inch plate be employed—16 cc. or so must be prepared. (4) Tubes are easier to count than plates. They possess no corners; one is dealing with a relatively flat surface throughout, whereas with plates there is frequently an undesirable amount of spreading of the colonies at the junction of the bottom with the sides which renders counting a difficult and uncertain procedure. (5) Tubes can be incubated immediately after rolling, instead of having to be kept immobile for twenty minutes till the agar is set, as in the case of plates. Where time is a consideration the practical value of this point will be appreciated.

After finding that tubes were perfectly well suited for the purpose of performing the viable count, it was necessary to compare the results obtained with those obtained by means of plates. For this purpose two distinct sets of experiments were undertaken, differing in the way in which the plates were poured. In the first series viable counts were made on various broth cultures employing both tubes and plates. The tubes were put up and rolled in the way already described. In preparing the plates the emulsion to be counted was delivered into a test tube containing 15 cc. of melted agar which was then poured into a plate and allowed to solidify. Each was incubated for three days before counting. In all cases the conditions were strictly comparable. During the course of several experiments of this nature 69 tubes and 69 plates were examined. The total number of colonies in each set were added together: the results were as follows:

NUMBER OF COLONIES IN TUBES	NUMBER OF COLONIES IN PLATES	PERCENTAGE OF COLONIES IN PLATES TO THOSE IN TUBES
8741	7527	<i>per cent</i> 86.1

In reviewing these figures one was naturally struck by the distinct numerical inferiority of the plate colonies. It was surmised that part, at least, of this discrepancy might be attributable to the method of pouring, since in this procedure a certain unknown quantity of agar is bound to be left behind in the tube. To test this a second series of experiments was carried out, similar to the first, except that the drops of emulsion were delivered directly into the plate, and the agar poured on top of them, mixing being performed as well as possible by gentle tipping to and fro of the plate. In the course of three experiments a comparison of 14 tubes and 14 plates was made, with the following results:

NUMBER OF COLONIES IN TUBES	NUMBER OF COLONIES IN PLATES	PERCENTAGE OF COLONIES IN PLATES TO THOSE IN TUBES
8041	7636	<i>per cent</i> 94.93

Comparing these two sets of experiments it would appear that approximately 9 per cent of the contents of the tubes must be left behind when these are used for pouring agar into the plates. This is somewhat higher than the figure quoted by Winterberg, who likewise estimated this error, though in a different way: he found it to be about 5 per cent. At any rate it may be concluded that this method of inoculating plates does introduce a considerable error, the effect of which will be to give a uniformly lower count than should actually be the case. On the other hand it would be thought that when the emulsion is delivered directly into the plate, and the agar poured over it, the count should be the same as that yielded by the roll tube method; it is seen, however, from the second protocol that the plate count is still 5 per cent below the roll tube count. The explanation of this may lie in the possibility that too small a number of experiments was

made, but, while granting that this may be true, it would appear more probable that the difference is due chiefly to a lack of uniform mixing of the emulsion with the agar in the plate preparations. An examination of these showed that the colonies tended to be aggregated in groups, while in the roll tubes they were distributed equally throughout the agar. The effect of this crowding is, as is afterwards demonstrated, to lead to the failure of certain bacilli to develop into single colonies; hence a smaller number of colonies will result than would otherwise be the case when the bacilli are separated by a sufficient space from each other. If, in the plate preparations, uniform mixing could be insured, this difference would probably disappear; incidentally, however, the difficulty of insuring such a mixture is by no means imaginary, and whatever method be employed for gaining this purpose an unnecessarily large amount of time must be spent over each plate.

To sum up briefly, then, it is claimed that roll tubes possess certain advantages over plates, the chief of which is undoubtedly that the count tends to be higher—probably at least 5 per cent—and therefore presumably more accurate. The only objection to their use is that they take a slightly longer time to put up; this is more than counterbalanced, however, by the greater rapidity and ease with which they can be counted.

#### *The nature of the diluent*

For counting emulsions dilution has been preferred to direct sowing. For this purpose a suitable number of drops is delivered into a flask containing the diluent, and if necessary a further dilution is made by the use of a second flask. In every case a fresh pipette is used for each dilution.

In the early stages of this work attention was directed to the nature of the diluent used in preparing the emulsions for counting. A review of the literature shows that the most popular fluids employed for this purpose have been saline, distilled water, and tap water. A preliminary experiment undertaken to ascertain whether any material difference could be discerned between these

three diluents showed that the organisms remained alive longest in tap water, while in distilled water they rapidly died out. With regard to saline, Flexner (1907) found that a pure solution of sodium chloride was distinctly inimical to the life of the Meningococcus. More recently Shearer (1919), while confirming this result, pointed out that the deleterious action of this salt could to a certain extent be neutralized by the addition of a small amount of calcium.

To investigate this effect more closely, a saline emulsion of a five and one-half hours' broth culture of *Bact. suispestifer* was prepared, and roll tubes were put up from it at various intervals, 3 to 5 tubes being employed for each count. The emulsion was put up at 3 p.m., and the counts were made each hour till 6 p.m., and again the following morning at 9.30 a.m. The results were as follows:

TABLE 2

TIME	COUNT PER CUBIC CENTIMETER
3 p.m.	125,500,000
4 p.m.	120,400,000
5 p.m.	101,100,000
6 p.m.	45,380,000
9.30 a.m.	Sterile

"Sterile" = no organisms developing in a tube seeded with 0.1 cc. of the emulsion.

The effect, then, of eighteen and one-half hours' contact of the bacilli with saline, was to sterilize the emulsion. Considering that this effect might be due to absence of certain salts in the diluent which were necessary for the continued viability of the organisms, comparisons were instituted between tap water, saline, and Ringer's solution. Three sets of two flasks were taken, and equal quantities of these three fluids placed in them, respectively, in this order. The primary and secondary dilutions were made as nearly simultaneously as possible. After performing a viable count on each of these secondary dilutions, the flasks were lightly plugged with cotton wool to protect them from contaminating organisms. Viable counts were made at intervals up to twenty-

one hours on each of them. The results have been plotted in the form of a graph to render them more striking (fig. 1). The figures are given in terms of the original culture. From this it will be seen that while all three fluids have a deleterious effect on the organisms, that of Ringer's solution is the least marked, and saline the most pronounced, that of tap water occupying an intermediate position.

A fresh experiment was performed with the idea of discovering what proportion of Ringer's solution was necessary to prevent

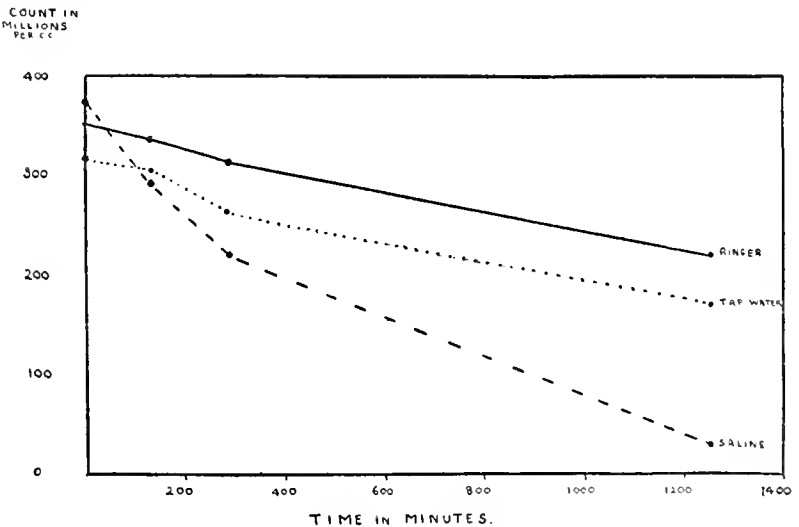


FIG. 1. SHOWING THE EFFECT OF VARIOUS DILUENTS ON THE VIABILITY OF BACTERIA

the deleterious effect of distilled water on organisms emulsified in it. A quantity of Ringer's solution was prepared according to the following formula:

Sodium chloride.....	0.9 grams
Potassium chloride.....	0.042 gram
Calcium chloride.....	0.048 grams
Sodium bicarbonate.....	0.02 gram
Distilled water.....	100.0 cc.

From it five separate dilutions were made with distilled water, commencing with one-half and passing by geometrical progres-



sion up to one in thirty-two. A three and one-fourth hour broth culture at 37°C. of *Bact. suispestifer* was used for counting and dilutions were put up almost simultaneously in the six fluids at hand. Viable counts were conducted at the start and thereafterwards at intervals up to 49 hours. In all cases the counts were performed under strictly comparable conditions. The results are shown in table 3, the counts being given in terms of the original culture.

Neglecting the slight irregularities in the series, which were probably due to small differences in the dropping pipettes used—this experiment being performed before a micrometer had been obtained for checking their calibration—it is at once clear that

TABLE 3

TIME	RINGER CONCENTRATION					
	Pure	1:2	1:4	1:8	1:16	1:32
<i>hours</i>						
0	109,200,000	112,800,000	101,900,000	111,700,000	98,830,000	87,770,000
3½	94,190,000	91,680,000	101,200,000	66,720,000	72,430,000	1,962,000
21	27,830,000	27,650,000	28,010,000	27,110,000	31,930,000	Sterile
27¼	28,910,000	19,270,000	9,633,000	12,490,000	19,630,000	Sterile
48½	15,310,000	2,855,000	1,427,000	2,141,000	3,925,000	Sterile

up to twenty-one hours the 1:16 dilution of Ringer's solution is no more harmful to the bacilli than the pure solution. But between the 1:16 and the 1:32 dilution there is an enormous difference; the latter acts just like distilled water, rendering the emulsion sterile within twenty-four hours. From this it may be inferred that a minute but quite definite amount of various kinds of salts is necessary to prevent the deleterious action of distilled water. What particular quantities of what particular salts was not entered into more fully, as it lay outside the scope of this work. The practical conclusion to be drawn from these experiments seems to be that in preparing dilutions for a viable count on a bacterial culture, the best fluid to use, so far investigated, is undoubtedly Ringer's solution; particularly is this the case if the emulsions have to be kept for some time, or possibly shaken, before the actual agar is inoculated. If the tubes are to be put

up within five or ten minutes of preparation of the dilutions, it is immaterial whether tap water, saline, or Ringer's solution be employed. For most purposes in this work tap water, sterilized by autoclaving at 120°C. for forty-five minutes, has been chosen as the diluent, and been found perfectly satisfactory. Just recently however, during the drought of June and July, it was noticed that the three tubes put up for each count during the course of routine work showed a marked disagreement with one another, and, on investigation this was referred to the tap water. Whether any marked change was produced in the saline content as a result of the drought, or whether possibly some metallic contamination had gained entrance to the water, was not ascertained, but on replacing the tap water by Ringer's solution, the tubes again showed close agreement with one another.

#### *Nature of the medium*

The nutrient agar used was prepared from a three weeks' casein digest at 37°C. (Cole and Jordan Lloyd, 1917). After filtration it was diluted till it had an amino-nitrogen content—as determined by Sørensen's method of formol titration—of 0.08 per cent, this being found to be the most suitable proportion for the purpose. An addition of 2.5 per cent agar was then made—higher amounts being found to exert a deleterious action on the development of the organisms. As no advantage of washed over commercial agar could be substantiated for the growth of *Bact. suipestifer*—a result which failed to confirm the findings of Ayers, Mudge and Rupp (1920) in the case of milk bacteria—the latter was uniformly employed. The hydrogen-ion concentration of the finished medium was allowed to vary from pH 7.4 to pH 7.8.

#### *Length of incubation and effect of moisture during incubation*

With regard to the length of time that the tubes should be incubated, experiments were made in which the number of colonies was counted on successive days up to a week. The result was to show that though the majority of the bacilli grow within the first twenty-four hours at 37°C., a few colonies

do not appear till the second or third days after inoculation. Later than this no further numerical increase was observed.

*The effect of shaking on broth cultures*

In view of the fact that the discrepancy in the relation of the viable to the total count has frequently been attributed to clumping of the organisms, leading to the development of a single colony from one mass of organisms instead of to several, and in view of the fact that the aim in this work was to eliminate every factor hindering the development of the maximum number of colonies, it was decided to investigate the effect of shaking on the cultures before the counts were put up. Shaken and unshaken cultures were compared, both total and viable counts being performed on them, the conditions being rendered identical except for the single process of shaking. As a result of several experiments on broth cultures of different ages it appeared that shaking in the case of a broth culture under twelve hours old led to no increase in either count; in a twenty-four hour culture, however, an increase of somewhere in the neighbourhood of 15 to 25 per cent could be substantiated in both. In the estimation of the count in young cultures, therefore, shaking is of no value: for the estimation of the count in older cultures, shaking leads to an absolute increase in both total and viable counts, but as the relation between the two remains unaltered, it is of no advantage so long as it is only this ratio which is being investigated.

*The optimum number of colonies per tube*

Speaking of the technique of the plate count Park and Williams (1908) remark:

It is very important to remember that when more than 200-300 bacteria start to develop in the agar or gelatin contained in a plate some develop colonies which fuse together, while others are inhibited before they develop visible colonies. . . . If possible, dilutions should be made so that plates will contain between 40 and 400 colonies.

This is one of the few references I have been able to find to the

effect of overcrowding in the determination of the numerical development of colonies in plates. However, in considering this question it must be realized that two factors are concerned. The first may be called the error of sampling, the second the error of overcrowding.

*a. The error of sampling.* It is clear that if three tubes are put up from an emulsion containing a comparatively small number of bacilli, the chances of obtaining a representative sample must be smaller than if an emulsion be employed which contains a much larger number of bacilli. Similarly with the tubes themselves. If only a few bacilli are introduced, the chances of obtaining a correct idea of the exact number are smaller than if a large number of bacilli are introduced. Thus the greater the number of colonies per tube, the less is the error of sampling. That this is not a mere theoretical consideration is shown from an examination of the data accumulated during the progress of his work. Thus to quote from the actual figures of an experiment. Three tubes were put up from a particular emulsion: in the first, 7; in the second, 12; and in the third, 15 colonies developed. The arithmetic mean of these three is 11.33, and the percentage mean deviation is 25.5 per cent. Or again; in a series of three tubes inoculated from the same dilution, the number of colonies developing was 434, 454 and 470. The arithmetic mean of these is 452.7, and the percentage mean deviation is 2.75 per cent. From these two examples it is seen that the percentage deviation of each individual tube from the arithmetic mean was considerably greater in the case when a small number of colonies developed than in the case when a large number of bacilli were inoculated. In other words the sampling error in the former instance was large, in the latter comparatively small. Recognizing however, the fallacies of a single experiment, a large number of experiments were reviewed in which viable counts were put up by means of three tubes each; the arithmetic mean of each of these counts was worked out, and the percentage deviation of each count from the arithmetic mean estimated. The percentage mean deviation was calculated for tubes in which 0 to 50 colonies developed, and again for those in which 50 to 100, 100 to 200, 200 to 400, 400 to

800, and 800 and over developed. The results are shown in table 4.

These figures, of course, do not represent the sampling error itself; this can only be obtained by putting up comparable series of tubes from the same emulsion; but they do show that the fewer colonies there are per tube, the less chance is there of obtaining an accurate sample of the emulsion under investigation. When more than 800 develop, the increasing accuracy of representation is overshadowed by the error introduced by the difficulty experienced in the actual counting of the tubes. The conclusion may therefore be drawn that in order to keep the sampling error as low as possible, somewhere between 400 and 800 bacilli should be inoculated per tube. Naturally, if more than three tubes are put up, the sampling error will be smaller, but as

TABLE 4

	0 TO 50	50 TO 100	100 TO 200	200 TO 400	400 TO 800	800 AND OVER
Number of experiments.....	13	24	43	42	42	11
Percentage mean deviation.....	19.6	9.42	7.13	6.08	4.44	6.89

three has been selected as a suitable number for this work, the results have been calculated in accordance with this figure.

*b. The error of overcrowding.* We now come to consider the second factor determining the optimum number of bacilli to be inoculated in putting up viable counts by the tube method, namely the error of overcrowding. More or less in proportion as the error of sampling decreases as the number of developing colonies increases, so the error of overcrowding increases as the number of developing colonies increases. The two vary in opposite directions; the greater the number of colonies the less the sampling error; the fewer the number of colonies, the less is the overcrowding error. A point must be chosen between the two which will permit of the minimum combined error being experienced. Before this could be done however, it was necessary to ascertain the actual effect of overcrowding on the development of colonies in tube preparations. As mentioned above, this overcrowding error is one which seems to have been neglected by the majority of

observers, or at any rate, not clearly recognized. It is obvious that the greater the number of bacilli distributed in a given space, the less is the interval between each of them, and the greater the chance of two being coincident. In every case in which two bacilli are coincident or are placed very close together only one colony will develop. Further, when two bacilli are situated at such a distance from each other that each is able to develop, yet at such a distance that continued development of both will result in fusion, it is clear that a single colony must arise. Whether one continues to grow and the other desists or whether both develop, the result must be the same—namely, the appearance of one colony in place of two bacilli. On pure *à priori* grounds one would expect this overcrowding factor to be of considerable importance in determining the number of colonies which will develop in a given space. One would expect it to play but a small part so long as comparatively few bacilli were inoculated, but as the number of the latter increased so should the percentage which fails to develop into colonies become greater. So much for theoretical considerations. It was decided to make a quantitative estimation of this overcrowding factor and the following experiments were performed. A dilution in tap water of a 3 hours' broth culture of *Bact. suispestifer* was put up, of such strength that there was approximately one bacillus in every drop. A series of agar tubes was then inoculated with varying numbers of drops, the tubes being put up in such an order as to equalize the possibly deleterious action of the diluent. As a rule in each experiment, 16 to 20 tubes were inoculated with one drop, 6 to 10 with 5 drops, 6 with 10 drops, 4 with 15 drops, and 4 with 20 drops. After three days' incubation the tubes were counted, and the actual number of colonies developing in the different sets of tubes compared. It is clear that if one colony developed per tube when 1 drop was inoculated, then 5 should develop in the 5-drop tubes, 10 in the 10-drop tubes, 15 in the 15-drop tubes and 20 in the 20-drop tubes. After a number of experiments had been performed with emulsions containing from 1-15 bacilli per drop, another series was instituted in which the emulsions contained from 15 to 30 bacilli per drop and later on, a further series

in which the emulsions contained as many as 30 to 170 per drop. In all 26 experiments were performed. In each experiment the number of colonies which developed in the 5-, 10-, 15-, and 20-drop tubes was calculated as a percentage of the number which developed in the 1-drop tubes, after they had been rendered comparable with these by dividing by the number of drops they respectively contained. An example will make this clear: the number of colonies given represents the arithmetic mean of the counts of the number of tubes used:

TABLE 5

	NUMBER OF COLONIES IN TUBES CONTAINING				
	1 drop	5 drops	10 drops	15 drops	20 drops
Actual count.....	77.0	371.0	690.8	918.8	1173.8
Count divided by number of drops per tube...	77.0	74.2	69.08	61.25	58.69
Percentage of 1-drop counts.....	100	96.36	89.71	79.56	76.23

Here it is seen that the greater the number of organisms inoculated per tube the lower is the actual percentage which develops. In the 1-drop tubes, 77 colonies develop; theoretically therefore, in the 20 drop tubes 1540 colonies should have appeared, but in point of fact only 1173.8 were observed; that is, only 76.23 per cent of the bacilli inoculated formed colonies. The results of each experiment were plotted as a curve, the number of colonies per tube being arranged along the abscissae, and the percentage which developed along the ordinates. In the later curves, starting, for instance, with 100 colonies in the 1-drop tubes, the base could not be taken as 100 per cent, but had to be brought into relation with the results of the earlier curves; reference to these showed that in a tube containing 100 colonies only 97.6 per cent of the bacilli inoculated had developed, and therefore this figure was taken as the starting point for the curve, the other figures in the curve being taken as percentages of this number. Of course, in the earlier curves, when only a few bacilli were being dealt with per tube, the results were very irregular owing to the large sampling error involved; it was hoped that by taking the arithmetic mean of the counts in 16 to 20 tubes, this would be obviated, but

such was not altogether the case. But as the irregularity affected the curves more or less equally in the opposite directions, it is believed that the results obtained by combining all the curves into one composite curve represent the actual facts with a certain degree of accuracy (fig. 2). An examination of this composite curve constructed from the 26 simple curves involved shows that the overcrowding error does not come into play till about 50 bacilli are inoculated per tube. After this point the curve decreases gradually, proceeding obliquely downwards in almost a straight line. Had it been continued far enough, no doubt the obliquity of the descent would have decreased, otherwise at a certain point it would have reached zero, which would manifestly have been absurd. More than 1200 colonies were not counted, as above this point the error due to counting becomes so considerable as to invalidate the results obtained. Indeed any number of colonies greater than 800 per tube renders counting a distinctly arduous procedure, and the curve was continued beyond this point more for theoretical than for practical reasons.

We may now return to the optimum number of colonies desired per tube. It was seen that the sampling and the overcrowding errors varied in opposite directions, and it was therefore necessary to obtain a number which should permit of the minimum error of both. But now that the overcrowding error has been worked out in the form of a curve, it is possible to correct one's results in accordance with this curve, so that this error may be left out of account when the optimum number of colonies per tube is considered. The only factors influencing this point now are the sampling error and the actual error of counting. It was shown before that if only three tubes are used for each count, in order that the deviation of each tube, from the arithmetic mean may not exceed 5 per cent, between 400 and 800 colonies per tube should be aimed at. The counting error is naturally smallest when the colonies are fewest, but it is of little import till the number per tube rises to about 400. This error has been ascertained by counting the same tube twice in succession. The error involved is remarkably small, and not until something like 800 to 1000 colonies are counted does it become appreciable. The time,



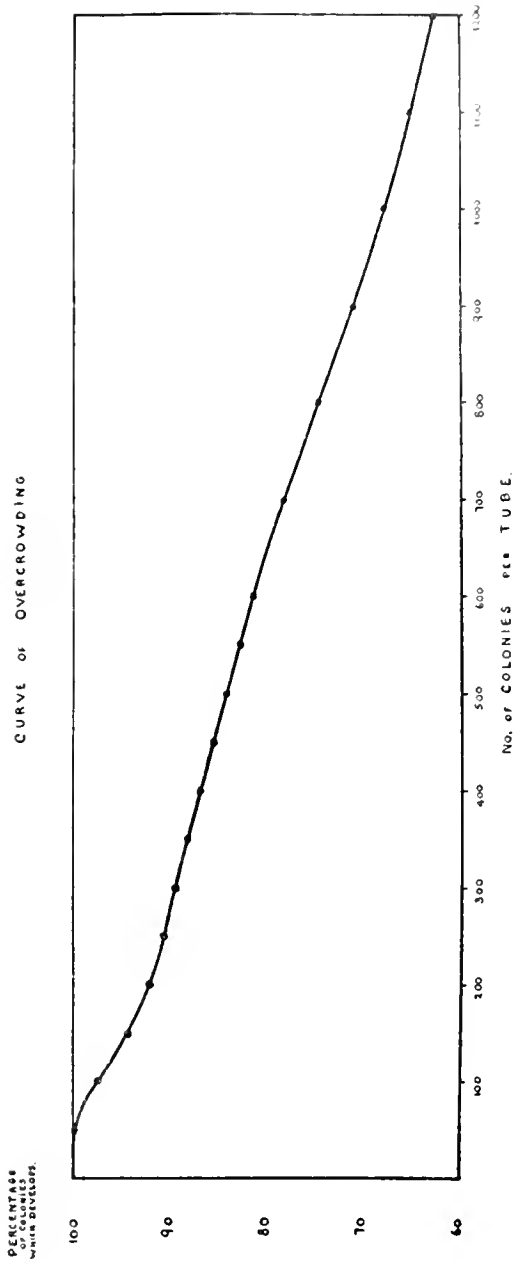


FIG. 2

however, required to count tubes containing this number of colonies is considerable, if several tubes have to be dealt with, and the resulting gain is comparatively small. Taking all factors into consideration then, it would seem that if considerable accuracy is required the optimum number of colonies to be desired per tube is in the neighborhood of 200 to 400, when only three tubes are put up; if a greater number are prepared, the number of bacilli inoculated may be decreased to 100. This will be seen to be somewhat higher than the number advocated by Park and Williams, but taking the experimental data given above as a guide, one feels justified in regarding this recommendation as more in accordance with the laws of scientific accuracy.

#### *Technique of the viable count*

Having dealt with these preliminary points, the final technique of the viable count may now be described. Presuming a broth culture of an organism is to be counted, a certain amount of fluid is withdrawn by means of a dropping pipette, and 10 drops are delivered at intervals of one second between each drop into a flask containing a known quantity of sterile tap water at 18°C. After shaking thoroughly, a fresh pipette is used to transfer 10 drops of this emulsion into a second flask, likewise containing a known quantity of tap water at 18°C. The quantity of water in each flask will vary according to the age of the culture which is to be counted; in some cases only 5 cc. will be required, in others as much as 50 or 100 cc.; in any case, the quantity is delivered by means of a carefully calibrated volumetric pipette. When the second flask has been shaken 4, 8 or 12 drops of this emulsion are delivered into three test tubes, measuring 6 inches by  $\frac{5}{8}$  inch, each containing about 2 cc. of melted agar at 45°C. The contents are then mixed by gentle shaking, and the tubes are rolled by rotation between the fingers. The tubes are incubated in an inverted position for three days at 37°C., and at the end of that time are counted in the way previously described.

*The error of comparable dilutions*

In order to form an estimate of the actual error of the complete process, 8 experiments were performed in each of which two separate counts of the same emulsion were made under exactly comparable conditions. In each count three tubes were put up and the arithmetic means of these were compared, and the percentage deviation of each set from the arithmetic mean of the two was calculated. The results are shown in table 6.

The results are sufficient to show that the error involved in the viable count performed by the method described is probably

TABLE 6

EXPERIMENT NUMBER	COUNT OF FIRST SET	COUNT OF SECOND SET	ARITHMETIC MEAN OF TWO SETS	PERCENTAGE DEVIATION
				<i>per cent</i>
1	229,000,000	203,500,000	216,300,000	5.92
2	228,400,000	229,800,000	229,100,000	0.306
3	339,500,000	393,100,000	366,300,000	7.31
4	198,200,000	196,200,000	197,200,000	0.50
5	491,500,000	483,900,000	489,200,000	1.08
6	187,800,000	188,000,000	187,900,000	0.053
7	342,100,000	302,000,000	322,100,000	6.24
8	423,500,000	427,700,000	425,600,000	0.049
Mean percentage deviation .....				2.68

somewhere in the neighborhood of 5 per cent. For practical purposes this seemed to be sufficiently small to render the method applicable to the study for which it was designed.

Before passing on, however, it is interesting to note that the actual counts obtained of the number of living organisms in a broth culture of *Bact. suipestifer* have been found to be considerably higher than those of many previous observers. Penfold, for instance, working with *Bact. coli* in peptone water, records a maximum of not more than 9,500,000 organisms per cc., while by the method employed in this work, counts of over 700,000,000 organisms per cc., have been met with. Whether such a difference can be ascribed merely to the nature of the medium and to the method employed in counting must be left an open point.

## PART II

*Comparison of the total and viable counts*

The primary object of this work was to make a comparison of the total and viable counts, especially during the logarithmic phase of growth, in order to ascertain if they were equal. From a survey of previous work on the subject, one was led to expect that all the bacilli would be found to be alive during this period. To test this a series of 16 experiments was made. At the start

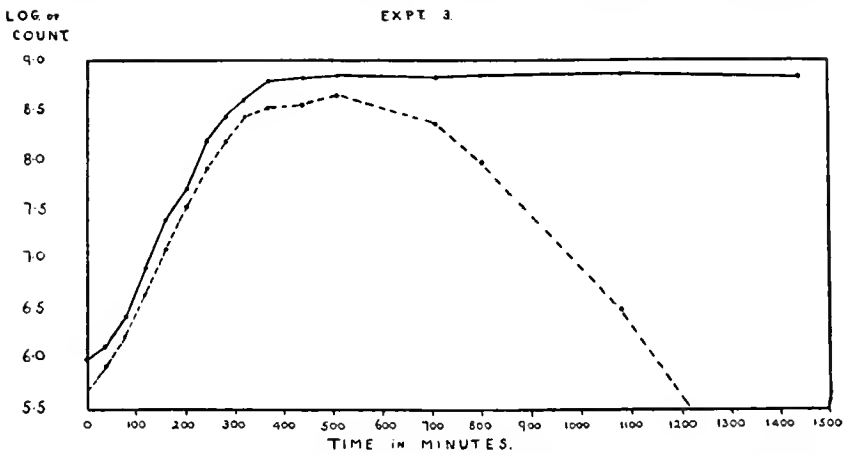


FIG. 3. SHOWING THE TOTAL AND VIABLE COUNTS IN A BROTH CULTURE OF *BACT. SUIPESTIFER*

Total count = continuous line. Viable count = interrupted line

comparative counts were conducted for the first 24 hours of growth so as to obtain an idea of the general relations of the two curves, but later on the counts were not continued beyond six to nine hours, as by that time the logarithmic phase was already past, and no object in following further progress was to be achieved.

To take one of the first experiments. The technique was as follows: At 6 p.m., in the evening a tube of 5 cc. of broth was inoculated with one loop of a culture of *Bact. suispestifer*, and incubated at 23°C. overnight. At 9.20 a.m. the following morning, after being warmed to 37°C., 10 drops of this culture were used

to seed a flask of 50 cc. of broth also warmed to 37°C. A total and viable count was made on the old culture, and from this the actual number of bacilli, alive and dead, in the inoculum was calculated. Unfortunately it was only found possible to make one determination of the total count; had three been made and the arithmetic mean taken a closer approximation to the real count would have been obtained. After thorough slaking, the

TABLE 7  
*Experiment 3*

TIME AFTER INOCULATION	VIABLE COUNT PER CUBIC CENTIMETER	TOTAL COUNT PER CUBIC CENTIMETER	RELATION OF VIABLE TO TOTAL
<i>minutes</i>			<i>per cent</i>
0		962,300	
40	828,800	1,286,000	64.46
80	1,539,000	2,574,000	59.84
120	4,363,000	8,123,000	53.71
160	12,150,000	23,970,000	50.70
200	32,490,000	49,650,000	65.45
240	81,640,000	155,300,000	52.51
280	155,900,000	280,600,000	55.67
320	271,700,000	401,400,000	67.70
370	334,300,000	606,000,000	55.16
440	351,400,000	669,500,000	52.47
510	454,000,000	693,300,000	65.49
710	223,700,000	647,900,000	34.52
800	91,950,000	709,600,000	13.39
1080	3,134,000	702,300,000	0.45
1440	7,709	658,800,000	0.001

flask was incubated at 37°C., and total and viable counts were made on it at intervals during the ensuing twenty-four hours. The early total counts were made by the Thoma-Zeiss slide, using methylene blue for staining the organisms, and examining the preparations under open illumination; the later counts were made in the usual manner with the Helbe under dark ground illumination. The results were as shown in table 7 and figure 3.

The irregularity of the total count during the first three hours will be noted. This was due to the unsatisfactory method employed. Until the count reaches about fifty million per cubic

centimeter, when the Helbe chamber can be used, there appears to be no method of insuring accuracy in the results. As variations of 50 per cent and more were encountered by the Thoma-Zeiss method it was decided to abandon this altogether and wait till the numbers were sufficiently great to allow the Helbe to be used. In future experiments, then, no record of the total counts

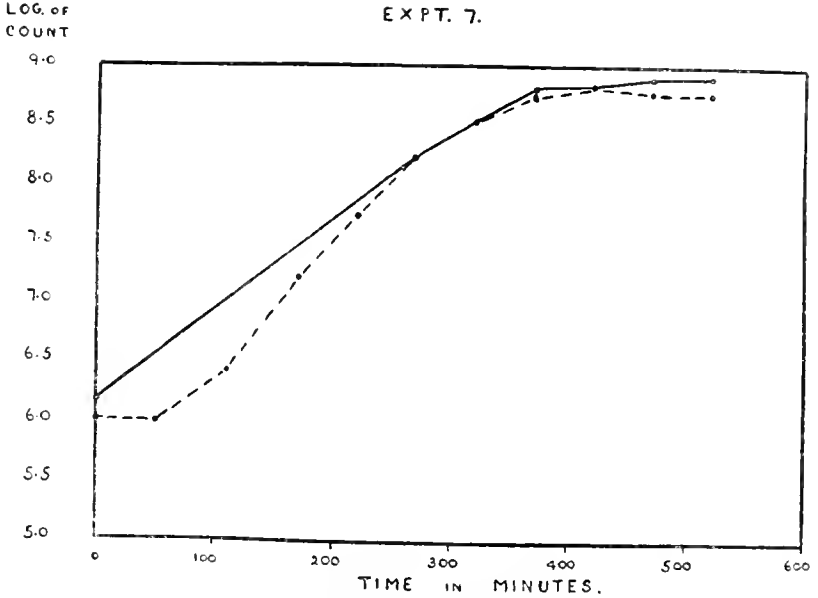


FIG. 4 SHOWING THE TOTAL AND VIABLE COUNTS IN A BROTH CULTURE OF BACT. SULPESTIFER

Total count = continuous line. Viable count = interrupted line

during the early phases was made. There is no doubt that this is a grave disadvantage, for it prevents one from obtaining figures by which to compare the total and viable counts in the early stages of the logarithmic phase. In practice it was only found possible to obtain one to three comparative counts before the close of the logarithmic phase. As, however, the organisms are dividing more or less uniformly during this phase, it is fair to assume that the relation between the two during the whole of the phase is fairly closely represented by that obtaining during the

latter part. Referring once more to the graph it will be seen that during no part of the growth does the viable count equal that of total. The relation of the two worked out as percentages of the viable to the total is given in the third column of the protocol.

Actually at the end of the logarithmic phase, at two hundred minutes, the relation will be seen to be one of 65.45 per cent. As it has previously been shown that the probable error of either of the counts is not more than about 5 per cent, any question of technical defects falls to the ground.

TABLE 8  
*Experiment 7*

TIME AFTER INOCULATION	VIABLE COUNT PER CUBIC CENTIMETER	TOTAL COUNT PER CUBIC CENTIMETER	RELATION OF VIABLE TO TOTAL
<i>minutes</i>			<i>per cent</i>
0	1,021,000	1,486,000	68.95
50	979,700		
110	2,631,000		
170	16,590,000		
220	54,730,000		
270	169,100,000	162,100,000	104.3
320	335,500,000	336,700,000	99.63
370	581,500,000	669,500,000	86.84
420	693,300,000	691,500,000	100.2
470	626,600,000	813,000,000	77.07
520	603,400,000	825,600,000	73.08

To confirm this, other experiments were performed, the technique being kept constant except for the nature of the inoculum. This was varied in several ways to see whether the previous history of the culture from which the 5 cc. tube of broth was seeded, whether old, recent, or very young, had any effect on the subsequent growth. This was found not to be the case. Again, it was thought that possibly the discrepancy in the relation of the two might be due to the presence of a lag phase, but obliteration of this factor by inoculating the flask of broth from a young culture growing at 37°C. failed to substantiate this either. In other experiments the inoculum was from the last of a series of rapid subcultures of the organism, no subculture being allowed

to grow for more than three hours at 37°C., so as to maintain the organisms in a medium of comparatively fresh broth. This again was without effect. The viable count still persisted below the total. No method, in fact, could be ascertained which would abolish this inferiority, and it was thought that possibly the tech-

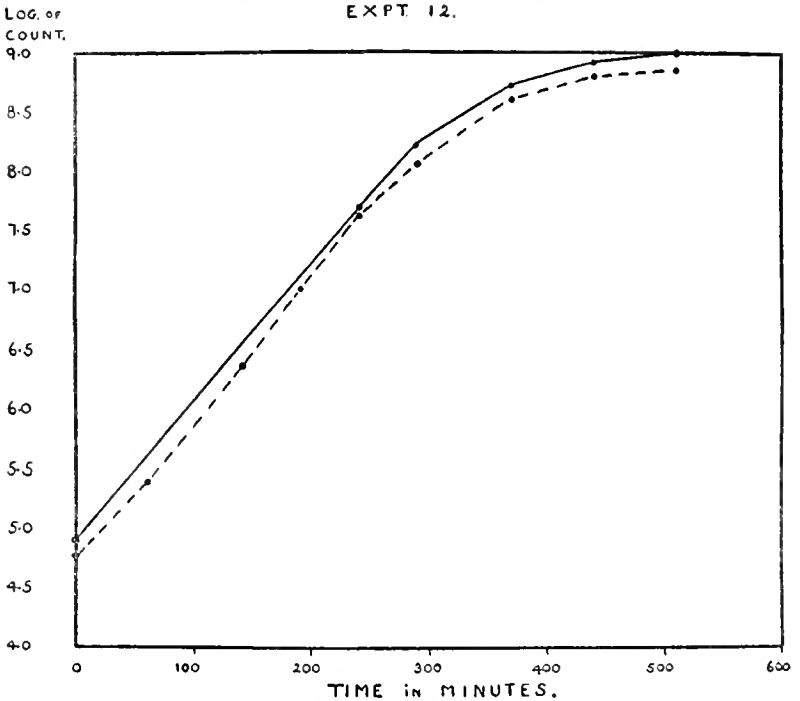


FIG. 5 SHOWING THE TOTAL AND VIABLE COUNTS IN A BROTH CULTURE OF BACT. SUIPESTIFER

Total count = continuous line. Viable count = interrupted line

nique or the calibration of the instruments employed might be at fault. But this idea had likewise to be given up when quite unexpectedly the result of one experiment showed that during at least fifty minutes of growth the two counts were identical. The figures for this experiment are quoted in table 8 and figure 4.

The low total count, at four hundred and twenty minutes was probably due to a technical error. Apart from this it will be seen



that the relation of the viable to the total count rises from 68.95 per cent at the start to equality; after remaining at this level for at least fifty minutes, it declines gradually to 73.08 per cent at 520 minutes. The reason for this equality was not forthcoming, but that it, too, was not due to a technical defect was shown by the fact that in three other experiments a similar equality of the total and viable counts was met with. No amount of analysis of these curves has yet succeeded in demonstrating the reason for the difference in the results between these four and the other twelve experiments. The previous history of the culture, the

TABLE 9  
*Experiment 12*

TIME AFTER INOCULATION	VIABLE COUNT PER CUBIC CENTIMETER	TOTAL COUNT PER CUBIC CENTIMETER	RELATION OF VIABLE TO TOTAL
<i>minutes</i>			<i>per cent</i>
0	62,150	81,470	76.28
60	249,700		
140	2,417,000		
190	10,570,000		
240	43,290,000	50,780,000	85.23
290	116,900,000	176,000,000	66.37
370	416,100,000	535,100,000	77.76
440	646,800,000	860,400,000	75.16
510	751,500,000	1,045,000,000	71.90

size of the inoculum, the relation between the total and viable counts in the inoculum, the presence or absence of lag, the duration of the logarithmic phase, the actual increase in numbers during the logarithmic phase, and the rate of growth have all been studied without showing any definite correlation between these factors in the 4 experiments quoted.

In conclusion one more example will be given in which at the end of the logarithmic phase the relation between the total and viable counts was as high as 85.23 per cent (table 9 and fig. 5).

Here it will be seen that the condition is intermediate between the two curves already shown. Unfortunately from lack of space it is impossible to reproduce the curves for each of the sixteen counts, but the individual protocols will be found in tables 10 to 22.

TABLE 10  
*Experiment 1*

TIME	TOTAL COUNT PER CUBIC CENTIMETER	VIABLE COUNT PER CUBIC CENTIMETER	PERCENTAGE OF VIABLE TO TOTAL
<i>minutes</i>			<i>per cent</i>
0	1,586,000	961,000	60.57
60		1,199,000	
120		6,337,000	
180	50,900,000	39,680,000	77.94
240	141,900,000	118,400,000	83.43
300	723,600,000	381,500,000	52.71
360	967,900,000	617,500,000	63.80

TABLE 11  
*Experiment 2*

TIME	TOTAL COUNT PER CUBIC CENTIMETER	VIABLE COUNT PER CUBIC CENTIMETER	PERCENTAGE OF VIABLE TO TOTAL
<i>minutes</i>			<i>per cent</i>
0	1,327,000		
50		1,350,000	
90		3,522,000	
130		10,320,000	
165	53,190,000	24,200,000	45.50
200	130,100,000	45,500,000	34.97
240	234,200,000		
295	360,300,000	241,600,000	67.05
360	628,700,000	509,300,000	81.00

TABLE 12  
*Experiment 4*

TIME	TOTAL COUNT PER CUBIC CENTIMETER	VIABLE COUNT PER CUBIC CENTIMETER	PERCENTAGE OF VIABLE TO TOTAL
<i>minutes</i>			<i>per cent</i>
0	108,400		
50		181,200	
100		446,300	
150		1,345,000	
220		8,846,000	
260	51,350,000	29,710,000	57.86
300	107,600,000	69,490,000	64.52
340	206,500,000	104,600,000	50.63
375	271,100,000	163,300,000	60.24

TABLE 13  
*Experiment 5*

TIME	TOTAL COUNT PER CUBIC CENTIMETER	VIABLE COUNT PER CUBIC CENTIMETER	PERCENTAGE OF VIABLE TO TOTAL
<i>minutes</i>			<i>per cent</i>
0	584,100		
40		972,900	
90		2,129,000	
132		4,506,000	
175		10,560,000	
220		34,430,000	
270	93,930,000	86,540,000	92.12
310	189,500,000	148,600,000	78.38
360	361,200,000	266,700,000	73.82
405	496,300,000	371,000,000	74.76

TABLE 14  
*Experiment 6*

TIME	TOTAL COUNT PER CUBIC CENTIMETER	VIABLE COUNT PER CUBIC CENTIMETER	PERCENTAGE OF VIABLE TO TOTAL
<i>minutes</i>			<i>per cent</i>
0	959,600	913,500	95.19
50		1,495,000	
100		2,530,000	
150		7,951,000	
200		19,780,000	
250		68,100,000	
300	(?) 119,300,000	146,700,000	(?) 122.80
350	227,700,000	229,900,000	100.90
390	417,000,000	328,300,000	78.74
450	627,900,000	464,300,000	73.94

TABLE 15  
*Experiment 8*

TIME	TOTAL COUNT PER CUBIC CENTIMETER	VIABLE COUNT PER CUBIC CENTIMETER	PERCENTAGE OF VIABLE TO TOTAL
<i>minutes</i>			<i>per cent</i>
0	1,851,000	1,692,000	91.41
50		1,852,000	
110		2,101,000	
170		6,548,000	
220		24,740,000	
270	76,630,000	49,140,000	64.12
320	(?) 252,400,000	102,400,000	(?) 40.58
370	365,100,000	230,700,000	63.19
420	526,200,000	361,400,000	68.68
470	771,300,000	584,500,000	75.78

TABLE 16  
*Experiment 9*

TIME	TOTAL COUNT PER CUBIC CENTIMETER	VIABLE COUNT PER CUBIC CENTIMETER	PERCENTAGE OF VIABLE TO TOTAL
<i>minutes</i>			<i>per cent</i>
0	726,300	501,800	69.08
50		892,300	
110		2,460,000	
170		7,802,000	
220		18,410,000	
270	75,900,000	59,570,000	78.48
320	167,100,000	131,500,000	78.66
370	361,200,000	235,100,000	65.09
420	564,400,000	423,100,000	74.96
470	653,300,000	490,500,000	75.08

TABLE 17  
*Experiment 10*

TIME	TOTAL COUNT PER CUBIC CENTIMETER	VIABLE COUNT PER CUBIC CENTIMETER	PERCENTAGE OF VIABLE TO TOTAL
<i>minutes</i>			<i>per cent</i>
0	1,020,000	869,800	85.33
50		1,409,000	
110		2,944,000	
170		11,700,000	
220		26,650,000	
270	89,780,000	66,160,000	73.69
320	238,900,000	135,400,000	56.67
370	388,600,000	287,200,000	73.91
420	533,400,000	430,300,000	80.67
470	854,700,000	558,600,000	65.36

TABLE 18  
*Experiment 11*

TIME	TOTAL COUNT PER CUBIC CENTIMETER	VIABLE COUNT PER CUBIC CENTIMETER	PERCENTAGE OF VIABLE TO TOTAL
<i>minutes</i>			<i>per cent</i>
0	1,303,000	1,198,000	91.89
50		1,699,000	
110		2,983,000	
170		12,480,000	
220	53,440,000	35,690,000	66.77
270	156,000,000	111,000,000	71.15
320	345,500,000	238,700,000	69.08
370	563,700,000	339,900,000	60.30
420	731,300,000	451,200,000	61.70
470	840,300,000	553,400,000	65.86

TABLE 19  
*Experiment 13*

TIME	TOTAL COUNT PER CUBIC CENTIMETER	VIABLE COUNT PER CUBIC CENTIMETER	PERCENTAGE OF VIABLE TO TOTAL
<i>minutes</i>			<i>per cent</i>
0	272,000	201,500	74.08
60		561,600	
140		5,141,000	
190		24,790,000	
240		121,100,000	
290	269,300,000	280,300,000	104.10
350	487,400,000	484,400,000	99.38
400	834,900,000	598,900,000	71.74

TABLE 20  
*Experiment 14*

TIME	TOTAL COUNT PER CUBIC CENTIMETER	VIABLE COUNT PER CUBIC CENTIMETER	PERCENTAGE OF VIABLE TO TOTAL
<i>minutes</i>			<i>per cent</i>
0	120,300	122,600	101.80
60		357,300	
140		2,094,000	
190		10,020,000	
230	46,260,000	29,270,000	63.27
250	64,600,000	50,340,000	77.94
270	89,190,000	67,670,000	75.88
350	364,100,000	273,200,000	75.02

TABLE 21  
*Experiment 15*

TIME	TOTAL COUNT PER CUBIC CENTIMETER	VIABLE COUNT PER CUBIC CENTIMETER	PERCENTAGE OF VIABLE TO TOTAL
<i>minutes</i>			<i>per cent</i>
0	132,000	85,920	64.94
130		3,045,000	
210	47,950,000	42,010,000	87.60
230	64,030,000	56,930,000	88.90
250	93,930,000	82,300,000	87.62
290	217,600,000	160,800,000	73.87
350	487,400,000	439,300,000	90.14

TABLE 22  
*Experiment 16*

TIME	TOTAL COUNT PER CUBIC CENTIMETER	VIABLE COUNT PER CUBIC CENTIMETER	PERCENTAGE OF VIABLE TO TOTAL
<i>minutes</i>			<i>per cent</i>
0	69,130	68,930	99.70
40		170,500	
160		5,585,000	
210		22,360,000	
225	55,440,000	39,090,000	70.52
240	(?) 76,740,000	69,950,000	(?) 91.16
260	111,100,000	81,850,000	73.69
280	196,700,000	116,500,000	59.23
390	522,600,000	417,100,000	79.80

## DISCUSSION

How, it may be asked, can the discrepancy between the total and viable counts during the logarithmic phase of growth be explained? It is only necessary to consider three hypotheses. (1) It might be referred to technical errors; that this is not so has been shown already. Moreover the uniformity of the individual counts suffices to alleviate one's few remaining suspicions on this point. (2) It might possibly be concerned with a method of reproduction other than that by binary fission. If, for instance, there were during certain phases of the culture a multiplication of the organisms by a process of budding or even by a sexual mode of some kind, it is conceivable that a certain number of parent cells might die or pass into a resting stage, and thus lead to the presence of a proportion of dead or inactive bacteria in the medium, even during the height of growth. For this, however, there is no direct evidence. Division of the particular strain of *Bact. suispestifer* used has been watched for several generations in gelatin film preparations on a large number of occasions, and never has any other method of reproduction than that by binary fission been encountered. (3) The most likely explanation would appear to be that of every generation produced a certain number of bacteria die. If, for example, 80 per cent of the organisms produced during a given generation continued

to live and divide, while 20 per cent failed to do so, the result would be that at the end of the logarithmic phase the total number of organisms alive and dead would exceed the number of living organisms. Further, the increase in the living organisms would still occur by geometrical progression, and the resultant curve of plotting the logarithms of the numbers would still fall on a straight line; the only difference would be that instead of the number of organisms being doubled in each generation, they would only be increased 1.6 times as much. To make this clear, suppose there are 1000 organisms per cubic centimeter, alive at the commencement of the logarithmic phase. At the end of the first generation there would be 2000 organisms, of which 80 per cent or 1600 would live and divide, while 20 per cent or 400 would

TABLE 23

	NUMBER OF VIABLE ORGANISMS PER CUBIC CENTIMETER	NUMBER OF DEAD ORGANISMS PER CUBIC CENTIMETER	TOTAL NUMBER OF ORGANISMS PER CUBIC CENTIMETER
At start.....	1,000	0	1,000
At end of first generation.....	1,600	400	2,000
At end of second generation....	2,560	640	3,200
At end of third generation.....	4,096	1,024	5,120

die. In the next generation these 1600 would divide and produce 3200 organisms of which again 20 per cent or 640 would die, while 2560 would live and divide and so on. In tabular form it may be seen more clearly still.

It will be noticed that the living organisms increase in each generation by 1.6 times as many as those present at the commencement. On the other hand the factors of increase for both dead and total organisms start high and gradually decrease till after several generations they approximate to 1.6. If curves were plotted of the logarithms of these counts, it would be seen that the curve for the viable organisms would lie along an ascending oblique straight line, whereas that for the total would rise at first rapidly and then gradually become flatter till it ran almost parallel to that for the viable count. Whether this is so in practice cannot be ascertained, as no practicable method of counting total numbers of organisms during the early part of the

logarithmic phase has been devised. So far, however, as the latter part of the phase is considered, it is seen that the total and viable curves do actually run very nearly parallel to one another. Whether or no this hypothesis be correct it does at least seem to explain how a dissociation of the total and viable counts may occur, and how the logarithmic curve for the viable count can yet ascend by means of a straight line—a fact which has been substantiated by several workers independently. Of course the actual percentage of the viable to the total is not constant, the figures in the 16 experiments undertaken varying from 57.86 to 122.8 per cent. Taking the arithmetic mean of the percentage relations between the two counts at the end of the logarithmic phase, the proportion in the 16 experiments came to 81.42 per cent. It seems justifiable to conclude therefore that under the ordinary conditions of in vitro cultures of *Bact. suis-pestifer* there is a normal death rate of approximately 20 per cent per generation, with variations extending between 43 per cent on the one hand to nil on the other. If this is actually so, it is a very surprising fact—totally in disaccord with all previous work on the subject. Translated into actual figures it means that no fewer than 200 organisms in every 1000 produced are dying in each generation. The old teaching has been accepted for so long—namely, that during the logarithmic phase all the bacilli are viable that one naturally feels diffident in contradicting it; yet the figures quoted seem to admit of no other course. Moreover, viewed from a general biological standpoint, it would be surprising if all the progeny in a given species continued to live and reproduce, however favorable the environment and however abundant the food supply. It seems to be very much more in accordance with the principles of this science that a certain proportion of each generation should fail to attain maturity and to propagate their kind; what the actual cause of this may be is difficult to ascertain, but the fact remains that in practically every species studied there is a variation in the progeny leading to the survival of some and the death of the others.



*Calculation of the generation time*

Taking for granted that there is a normal death rate of the organisms in vitro cultures, it is immediately apparent that the generation time must be calculated afresh. Hitherto the number of generations occurring during a given period has been calculated from the formula  $n = \frac{\log b - \log a}{\log 2}$ , where  $n$  = the number of generations,  $b$  = the number of organisms at the end of the given period, and  $a$  = the number alive at the beginning of that period (Chesney, 1916). This formula has been employed solely on the assumption that the number of organisms doubles in each generation. But if, for instance, only 80 per cent are dividing, then the increase in the number of organisms in each generation will only rise by 1.6. Hence the formula must be altered to  $n = \frac{\log b - \log a}{\log 1.6}$

The result of this will be to give a larger number of generations in the particular period studied, and consequently a shorter time for the production of each generation. It is now seen that the generation time cannot be calculated with accuracy unless the number of both the total and viable organisms be known. In these experiments these numbers are known, at least for the latter part of the logarithmic phase. What has been done then, is to estimate the relation between the total and viable organisms at the end of the logarithmic phase. Assuming that this relation has persisted all through the phase—and it is difficult to dispute the probability of this considering that the viable count increases regularly—one can then deduce the percentage of organisms which has remained alive and divided in each generation, and hence the total number of generations. For instance, reverting to the figures given for experiment 12 (table 9). The number of organisms alive at the commencement of the logarithmic phase, namely, 0 minutes, was 62,150 per cubic centimeter. At 240 minutes, the close of the logarithmic phase, there were 43,290,000 per cubic centimeter living and 50,780,000 per cubic centimeter total alive and dead. The proportion of viable to total is then 85.23 per cent. The actual increase in the viable count per

generation must therefore have been 1.71. Using the formula of  $n = \frac{\log b - \log a}{\log 1.71}$  it is found that in 240 minutes there were 12.2 generations, which gives a generation time of 19.7 minutes. Calculated by the old formula  $n = \frac{\log b - \log a}{\log 2}$  the number of generations is only 9.4, and the generation time is 25.5 minutes. It is clear therefore that the generation time is in general shorter than that hitherto recorded. To give an idea of the probable figure, the average generation times for the whole 16 counts during the logarithmic phases has been worked out; it comes to 21.05 minutes.

Taking all the facts into consideration, it seems that in cultures of *Bact. suispestifer* there is a normal death rate, even during the period when the maximum rate of growth is proceeding. The extent of this will vary in individual cultures. In some it is as high as 43 per cent, in others it is only 20 per cent or 10 per cent, while finally in a few it is for a short period actually nil.

#### CONCLUSIONS

1. A method for estimating the number of the total and the viable organisms in a culture medium is described. It is claimed that the experimental error involved in each count probably does not exceed 5 per cent.
2. Applying this to the study of the relation existing between the living organisms in a culture and the total number of organisms alive and dead, it is seen that even during the logarithmic phase the percentage of viable organisms seldom rises above 90 per cent of the total.
3. To explain this a hypothesis is advanced which supposes that in an *in vitro* culture of *Bact. suispestifer* there is a normal death rate amongst the bacteria, even during the logarithmic phase of growth.
4. Assuming the presence of a normal death rate, it has been possible to calculate the generation time on an altered basis, and

this time has been found to be shorter than that usually given for cultures of similar organisms.

It is with pleasure that I record my thanks firstly to Dr. W. W. C. Topley for his constant help and advice throughout the progress of this work, and secondly to Mr. J. E. Barnard, of the National Institute of Medical Research, for his assistance in the calibration of the Helbe counting chamber employed in these investigations.

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# A METHOD OF DETECTING RENNET PRODUCTION BY BACTERIA

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Received for publication January 19, 1922

The usual method for determining whether bacteria produce rennet or rennet-like enzymes is to inoculate the organisms under investigation into tubes of sterilized milk and then to notice whether or not the milk curdles without the production of acid. This method has several weaknesses. Heated milk is not as readily curdled by enzymes as unheated milk and the curd produced is often so soft that it can scarcely be detected; when an organism produces peptonizing enzymes as well, the presence of curd before peptonization is often difficult to demonstrate; certain organisms curdle the milk through the action of enzymes and subsequently bring about an acid reaction, so that it is difficult to tell whether the milk has been coagulated by the acid or by an enzyme.

It is possible by the following very simple method to avoid these weaknesses of the usual technic. Inoculate the culture under investigation into the milk in the usual manner; then incubate for twenty-four hours or such time as is necessary to allow the organism in question to produce vigorous action in the milk with at least 0.5 cc. of whey on the surface. At the end of this incubation period obtain fresh milk and place 10 cc. of it in a test tube, but do not sterilize it. Warm this milk to about 37°C. Then add to the milk a measured quantity, say 0.5 cc., of whey from the incubated culture and place in a 37° incubator. Examine every five minutes for the first half hour and, if not curdled then, at less frequent intervals for a few hours longer. If rennet is present in any abundance, the milk is ordinarily curdled inside of half an hour. By varying the quantity

of whey added to the milk from 0.1 cc. up to 1cc. and by recording the time necessary for coagulation, it is possible to make comparisons between different cultures on a quantitative basis.

The advantages of this method are as follows: the unheated milk curdles readily and forms a typical firm rennet curd; the peptonizing enzymes act more slowly and do not in any way obscure the curdling action of the rennet; the period of observation is so short that neither the bacteria present in the milk nor those added in the cultures have time to act on the milk themselves and the amount of whey added is too small to precipitate the casein by the action of any acid it might contain, hence the reaction occurring is due to the action of enzymes alone. By the use of this method it has been found possible to obtain typical rennet curds from certain organisms ordinarily producing both acid and rennet and from others ordinarily digesting the milk so rapidly that no true curd could be observed. By the usual method the production of rennet by these organisms was suspected but could not be actually demonstrated.

This technic is offered as an addition to the methods of pure culture study in use in connection with the society's descriptive chart.

# JOURNAL OF BACTERIOLOGY

## Contents

MAY, 1922—VOL. VII—No. 3

- J. HOWARD MUELLER. Studies on Cultural Requirements of Bacteria. I.  
J. HOWARD MUELLER. Studies on Cultural Requirements of Bacteria. II.  
SELMAN A. WAKSMAN. A Method for Counting the Number of Fungi in the Soil.  
LETHE E. MORRISON AND FRED W. TANNER. Studies on Thermophilic Bacteria.  
I. Aerobic Thermophilic Bacteria from Water.  
ROBERT G. GREEN. An Apparatus for the Rapid Measurement of Surface Tension.

MARCH, 1922—Vol. VII—No. 2

- F. C. HARRISON. Our Society.  
VICTOR BURKE. Notes on the Gram Stain with Description of a New Method.  
BARNETT COHEN. Disinfection Studies. The Effects of Temperature and Hydrogen Ion Concentration upon the Viability of *Bact. coli* and *Bact. typhosum* in Water.  
SELMAN A. WAKSMAN. Microorganisms Concerned in the Oxidation of Sulfur in the Soil. I. Introductory.  
SELMAN A. WAKSMAN AND J. S. JOFFE. Microorganisms Concerned in the Oxidation of Sulfur in the Soil. II. *Thiobacillus Thiooxidans*, a New Sulfur-oxidizing Organism Isolated from the Soil.  
E. B. FRED AND W. H. PETERSON. The Production of Pink Sauerkraut by Yeasts.  
CONSTANTINO GORINI. Studies on the Biology of Lactic Acid Bacteria: A Summary of Personal Investigations.  
L. D. BUSHNELL. A Method for the Cultivation of Anaerobes.  
L. D. BUSHNELL. Influence of Vacuum upon Growth of Some Aerobic Spore-Bearing Bacteria.  
H. R. BAKER. Substitution of Brom-Thymol-Blue for Litmus in Routine Laboratory Work.  
W. A. WALL AND A. H. ROBERTSON. The Use of Domestic Methylene Blue in Staining Milk by the Breed Method.

JANUARY, 1922—VOL. VII—No. 1

- HILDA HEMPL HELLER. Certain Genera of the Clostridiaceae. Studies in Pathogenic Anaerobes. V.  
O. ISHII. Studies upon Agglutination in the Colon-Typhoid Group of Bacilli.  
O. ISHII. A Study of Spontaneous Agglutination in the Colon-Typhoid Group of Bacilli.  
ALBERT C. HUNTER. The Sources and Characteristics of the Bacteria in Decomposing Salmon.  
S. A. KOSEK AND W. W. SKINNER. Viability of the Colon-Typhoid Group in Carbonated Water and Carbonated Beverages.  
GUILFORD B. REED. A Binocular Microscope Arranged for the Study of Colonies of Bacteria.  
An Investigation of American Stains.

NOVEMBER, 1921—VOL. VI—No. 6

- GEORGE E. HOLM AND JAMES M. SHERMAN. Salt Effects in Bacterial Growth. I. Preliminary Paper.  
HILDA HEMPL HELLER. Suggestions Concerning a Rational Basis for the Classification of the Anaerobic Bacteria. Studies in Pathogenic Anaerobes. IV. I. Preliminary Paper.  
J. HOWARD BROWN. Hydrogen Ions, Titration and the Buffer Index of Bacteriological Media.  
J. E. RUSH AND G. A. PALMER. On Decreasing the Exposure Necessary for the Gelatin Determination.  
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## CONTENTS

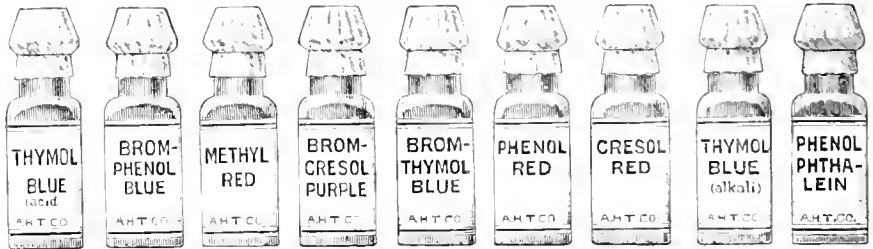
S. HENRY AYERS AND COURTLAND S. MUDGE. The Relation of Vitamines to the Growth of a Streptococcus.....	419
JAMES M. SHERMAN AND GEORGE E. HOLM. Salt Effects in Bacterial Growth. II. The Growth on <i>Bact. coli</i> in Relation to H-Ion Concentration.....	465
IVAN E. WALLIN. A Note on the Morphology of Bacteria Symbiotic in the Tissues of Higher Organisms.....	471
WILBURT C. DAVISON. Observations on the Properties of Bacteriolysants (D'Herelle's Phenomenon, Bacteriophage, Bacteriolytic Agent, etc.). Part I.....	475
WILBURT C. DAVISON. Observations on the Nature of Bacteriolysants (D'Herelle's Phenomenon, Bacteriophage, Bacteriolytic Agent, etc.). Part II.....	491
GEORGE F. REDDISH AND LEO F. RETTGER. <i>Clostridium putrificum</i> (B. <i>putrificus</i> Bienstock), a Distinct Species.....	505
J. HOWARD BROWN AND PAUL E. HOWE. Transparent Milk as a Bacteriological Medium.....	511
G. J. HUCKER AND W. A. WALL. The Use of Agar Slants in Detecting Ammonia Production and its Relation to the Reduction of Nitrates.....	515
H. J. CONN, K. N. ATKINS, H. J. BROWN, F. EBERSON, G. E. HARMON, G. J. HUCKER, F. W. TANNER AND S. A. WAKSMAN. Methods of Pure Culture Study. Report of Committee on Bacteriological Technique.....	519
H. J. CONN. An Investigation of American Gentian Violets. Report of Committee on Bacteriological Technique.....	529

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A comprehensive discussion of the whole subject from both the theoretical and practical standpoint is to be found in "*The Determination of Hydrogen-Ions*," I. r. W. M. Clark, Baltimore, 1920.



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# THE RELATION OF VITAMINES TO THE GROWTH OF A STREPTOCOCCUS<sup>1</sup>

S. HENRY AYERS AND COURTLAND S. MUDGE

*From the Research Laboratories of the Dairy Division, United States Department  
of Agriculture*

The importance of vitamins in animal nutrition has led to a little experimental work with bacteria. Results of various investigators have caused the belief that certain materials such as animal and plant tissues contain growth accessory substances which stimulate the growth of microorganisms and which with some bacteria are essential for growth.

Among the studies along this line may be mentioned the work of Cole and Lloyd (1917), Paccinni and Russell (1918), Hall (1918), Agulhon and Legroux (1918), Kligler (1919), Bachman (1919), Williams (1919), Willaman (1920), Davis (1921), Rivers and Poole (1921), and MacLeod and Wyon (1921). In some papers the growth-promoting substances are spoken of as growth-accessories substances, and in others as vitamins. The work in general clearly indicates that for some microorganisms there are present in certain materials growth-promoting substances. As long as this term is used, one is not committed to the assumption that these substances may be vitamins. Glucose in small amounts is a growth-promoting substance for many bacteria, in fact any easily available source of carbon can be considered a growth-promoting substance. If, however, the substances which have been found to be growth promoters are classed as vitamins, then there is a great possibility of investigators being misled as to the real connection between the recognized vitamins and the growth of microorganisms.

In this paper we shall present the results of a few experiments

<sup>1</sup> Presented at twenty-third annual meeting, Society of American Bacteriologists, Philadelphia, December 27, 1921.

22811 1922

with the water-soluble vitamin B and the fat-soluble vitamin A in their relation to the growth of a streptococcus.

#### EXPERIMENTS WITH WATER-SOLUBLE B

Throughout our experiments we have used a culture of a pathogenic streptococcus which grew slowly in a peptone yeast medium. This organism would not grow in a peptone medium without broth, and the fact that it would grow with the addition of yeast extract brought up the possibility that the water-soluble B might be the reason for the growth.

Several experiments were conducted in order to throw some light on this point. In the first experiment 10 grams of autolyzed yeast were extracted in a Soxhlet apparatus for eight hours with 95 per cent alcohol. Fresh alcohol was then added and the extraction continued for another eight hours. This process was repeated until the extraction had been continued for forty hours. During the extraction the yeast was removed several times from the thimble and the clumps were thoroughly disintegrated. As the alcohol was removed during the extraction it was evaporated at a temperature of 60° to 80°C. The combined residues from the successive extractions were added to 500 cc. of distilled water and heated for a half hour. To this was added an equal amount of 2 per cent Difco peptone and the reaction adjusted to pH 7.2. This medium was then steamed for fifteen minutes, filtered, placed in flasks, and sterilized. The residue from the original 10 grams of yeast which had undergone extraction by the 95 per cent alcohol was added to 500 cc. of distilled water and another medium prepared with Difco peptone as described. A third medium was prepared by using 10 grams of the regular autolyzed yeast which had not been extracted. This was added to 500 cc. of distilled water and a medium prepared with Difco peptone, the same as with the other media. In all the experiments the media were distributed in 50 cc. amounts in 100-cc. Erlenmeyer flasks. These flasks were approximately of the same shape so that the depth of the medium and the surface area exposed was about the same in each flask.

The flasks containing each of the three media described were inoculated with a twenty-four hour culture of the streptococcus and incubated at 37°C. Examinations for growth were made after seventeen, twenty-four and forty-eight hours. Assuming that the water-soluble B can be extracted by hot 95 per cent alcohol, the medium made up with the alcohol extract should

TABLE I  
*Growth of a streptococcus in various media*

MEDIA	HOURS OF INCUBATION			REMARKS
	18	24	48	
95 per cent alcoholic yeast extract + peptone .....	-	-	+*	Should contain water-soluble B
Extract from yeast residue + peptone.....	+*	+	++	Should not contain water-soluble B
Extract of regular yeast + peptone .....	+*	+	++	Contains water-soluble B
Fraction I + peptone.....	-	-	-	Should contain some water-soluble B
Fraction II + peptone .....	-	-	-	Should contain most water-soluble B
Filtrate A + peptone .....	-	+	++	Should contain little if any water-soluble B
Yeast extract treated with Lloyd's Reagent + peptone	-	+	++	Should not contain water-soluble B from yeast
Regular yeast extract + peptone.....	-	+	++	Should contain water-soluble B from yeast

\* Slight.

contain this vitamine. The medium prepared from the alcohol-extracted yeast should not contain the water-soluble B, or at least the amount should be greatly reduced. Further, the medium made from the regular yeast should of course contain the vitamine.

The results shown in table 1 were of considerable interest to us. The growth was recorded by + signs which were increased

in number as the growth seemed heavier. It will be observed that the growth was equally good in the medium prepared from the regular yeast and from the yeast extracted with the alcohol, whereas only a slight growth was manifest after forty-eight hours in the medium containing the alcoholic extract of yeast. The results would seem to indicate that water-soluble B is not the growth-promoting substance of yeast at least for the streptococcus used in this work.

There is, of course, a possibility that the water-soluble B was not soluble in the 95 per cent alcohol and was not contained in the extract, and for this reason another experiment was conducted in which a method similar to that of Osborn and Wakeman (1919) was used for the preparation of fractions containing different amounts of water-soluble B. Some of the same autolyzed yeast was used, 10 grams being added to 500 cc. of boiling water containing 0.01 per cent acetic acid. This was stirred for some time and filtered, the filtrate being concentrated to 300 cc. at a temperature of about 80°C. To this 300 cc. of filtrate sufficient 95 per cent alcohol was added to get a concentration of about 52 per cent. The solution was allowed to stand over night at about 5°C. and the precipitate which had appeared the next day was filtered off. This precipitate was known as *fraction I*. The filtrate was then concentrated to 200 cc. and sufficient 95 per cent alcohol added to give a concentration of 79 per cent. This solution also was allowed to stand over night at a temperature of 5°C., and the precipitate filtered off. This was known as *fraction II*. The filtrate was again concentrated, this time to 100 cc., and sufficient 95 per cent alcohol added to give a concentration of about 90 per cent. After standing over night at 5°C. the precipitate that formed was filtered off and kept as *fraction III*. The filtrate from fraction III, which we shall term *filtrate A*, was evaporated to dryness, and dissolved in 1000 cc. of distilled water, to which 1 per cent of Difco peptone was added, the reaction adjusted to pH 7.2 and the medium placed in flasks and sterilized in an autoclave. Fractions I and II were each dissolved in 1000 cc. of distilled water and 1 per cent Difco peptone was added to each lot. These media were adjusted, placed in flasks



and sterilized as described above. Osborn and Wakeman found that fraction II contained most of the water-soluble B, and that fractions I and III contained some. According to this we should expect that the peptone medium containing fraction II should contain most of the water-soluble B, that the medium containing fraction I should contain less, and that filtrate A should contain the least or none at all. On this assumption, that water-soluble B is the growth-promoting substance of yeast, it would be expected that media containing fraction II would give the best growth with the streptococcus. From the second section of table I it will be seen that such was not the case. There was no growth in peptone media containing fraction II or fraction I, but there was growth in the peptone medium containing filtrate A. These results seem to confirm those obtained in the first experiment and again indicate that water-soluble B is not the growth-promoting substance of yeast.

A third experiment was conducted in which the water-soluble B was considerably reduced and possibly entirely removed from the yeast extract. Seidell (1916) has shown that, by shaking autolized yeast extract with Lloyd's Reagent (Fuller's Earth) using 50 grams per liter of extract, only an inconsiderable amount of the vitamine appeared to remain in the filtrate. Ten grams autolized yeast were added to 200 cc. of distilled water. This was allowed to stand for about one hour with frequent shaking before filtering. To this filtrate 50 grams of Lloyd's Reagent were added together with HCl to make the solution 0.01 normal. The yeast extract was then shaken every half hour for four hours and the Lloyd's Reagent removed by filtration. The Lloyd's Reagent was supplied to us through the kindness of Dr. Seidell and was known to be an active grade of Fuller's Earth. The filtrate was made up to 1000 cc. to which 1 per cent of Difco peptone was added and the reaction adjusted to pH 7.2. After filtration this medium was put in flasks and sterilized in the usual way in the autoclave. Since it has been found that the Lloyd's Reagent removes a large part, if not all, of the water-soluble B from yeast extract it would be expected that a peptone medium containing a yeast extract treated with this substance would not

support growth of the streptococcus. The results shown in the third section of table I indicate that the growth in such a medium was just as good as the growth in our regular peptone medium containing the extract of autolized yeast.

We have only shown in our tables the result of growth in a single flask, but these results have been duplicated many times so that it is felt that the results presented express the general average.

The results of MacLeod and Wyon (1921) are interesting in connection with our experiments. They conducted some experiments on the growth of a streptococcus with extracts from various substances. They used extracts from liver, kidney, egg yolk, yeast, muscle, bran, and milk, and found that with the exception of milk and bran the results seemed to favor the vitamine hypothesis so far as small amounts were active. They found however, that yeast extract had but little more growth promoting power than muscle extract. In view of the high water-soluble B content of yeast, and of its small amount or even absence in muscle, these results appear to us again to indicate that the water-soluble B is not the growth-promoting substance of yeast.

MacLeod and Wyon also worked with the pneumococcus and meningococcus and they point out that the growth-promoting property of certain extracts did not bear a direct relation to the known vitamine content, and that yeast had little or no effect in promoting the growth of these organisms.

A survey of the second and third experiments shows that an attempt was made in two distinct ways to obtain data on the relation of water-soluble B to the growth-promoting substance or substances of yeast. In the second experiment accepted methods were employed for removing a fraction from yeast extract containing a large amount of water-soluble B. This fraction with peptone did not support growth of the streptococcus and this fact is very significant. In the third experiment accepted means were used for removing or at least greatly reducing the water-soluble B content of the yeast extract. This extract which should have been free or very nearly free from water-soluble B, with peptone, supported growth of the streptococcus in

a normal manner. The results of these experiments when interpreted in light of our present knowledge of water-soluble B, and applied to the streptococcus studied, permit only the conclusion that this vitamine is not the growth-promoting substance of yeast extract. It is interesting to note that Funk and Dubin (1921) working on the vitamine requirements of yeasts and bacteria, have isolated a substance which they believe to be definite and specific for the stimulation of the growth of yeast. This substance, which was separated from vitamine B, they call vitamine D.

#### EXPERIMENTS WITH CABBAGE EXTRACTS

It has been found that extracts from plant tissues apparently contain growth promoting substances for microorganisms, and cabbage seems to have given particularly good results. We tried one experiment in order to determine whether or not cabbage extract was valuable for the growth promotion of the streptococcus. One hundred cubic centimeters of finely minced cabbage were added to 300 cc. of distilled water and allowed to stand for forty-eight hours in the ice box. The suspension was then steamed for thirty minutes and filtered. Fifty cubic centimeters of 2 per cent Difco peptone solution were distributed into a series of flasks. To these flasks were added increasing amounts of the cabbage extract, these amounts being 1 cc., 5 cc., and 50 cc. Whenever necessary the medium was made up to 100 cc. and dispensed into tubes. Thus we had a series of media, one containing 1 per cent peptone alone and the other three having 1 per cent, 5 per cent, and 50 per cent of cabbage extract. Series of each of these media were inoculated with several different cultures (one of them being the culture used in the other experiments), were incubated for twenty-four hours, and then examined for growth and reaction. It was found that in the plain peptone without cabbage extract there was a fair growth of some of the streptococci, but with others the growth was questionable. In all of the tubes containing cabbage extract, growth was observed and the increase was more or less proportional to the increase in percentage of cabbage extract. The acidity also increased in a

similar manner. These results showed quite plainly that the cabbage extract contained some growth promoting substance, but the increase in acidity with the increase in percentage of cabbage extract also indicated that there was sugar present. This fact led us to consider the effect of sugar as a growth promoting substance. An analysis of the cabbage extract for which we are indebted to Doctor Rupp of these laboratories, showed that it contained 1.4 per cent of reducing sugar. Calculations showed that when the cabbage extract was incorporated with the peptone solutions the resulting media contained 0.014, 0.07 and 0.7 per cent of sugar for the 1, 5, and 50 per cent cabbage media, respectively. In order to show the effect of these percentages of sugar on the growth of the streptococcus a series of media was prepared as previously described except that a 1.4 per cent glucose solution was incorporated in the media instead of the cabbage extract. The growth in these last media was compared with that in the cabbage extract media mentioned.

From the results of this comparison, shown in table 2, it will be seen that growth in peptone media with cabbage extract was practically identical with that in the peptone media with the glucose solution. The growth increased, generally speaking, with the increase in the percentage of cabbage extract, and, in a similar manner, with the increase in the percentage of glucose solution. It should be remembered that the glucose solution contained 1.4 per cent reducing sugar so as to correspond with the cabbage extract. It is particularly interesting to note the increase in growth due to the incorporation of only 1 per cent of the glucose solution which gave a sugar content *in the medium* of only 0.014 per cent. From these results it seems evident that a very small amount of sugar acts as a growth-promoting substance for some of the streptococci.

We wish to emphasize that when extracts of plant tissues are used as a source of growth-promoting substances, the possible effect of sugar and other reducing substances present must be given considerable thought before the growth promotion can be attributed to vitamins. This is not only true when plant tissues are used but holds equally well for any extracts which may contain sugars.

TABLE 2  
*Effect on growth of streptococci of addition of cabbage extract and glucose to a peptone medium*

CULTURE NUMBER	NONE						1 PER CENT						5 PER CENT						50 PER CENT						
	Cabbage extract			Glucose solution			Cabbage extract			Glucose solution			Cabbage extract			Glucose solution			Cabbage extract			Glucose solution			
	Growth	pH		Growth	pH		Growth	pH		Growth	pH		Growth	pH		Growth	pH		Growth	pH		Growth	pH		
1	+	7.6	(?)	+	7.6		++	6.8		++	7.2		+++	6.2		+++	5.4		+++	6.0		+++	5.4		
2	+	7.6	+	7.6		++	7.0		++	7.2		+++	5.6		+++	5.4		+++	5.4		+++	5.4		+++	5.4
3	+	7.8	+	7.6		++	6.9		++	7.2		+++	4.8		+++	5.4		+++	4.5		+++	4.5		+++	4.5
4	+	7.4	+	7.4		++	6.8		++	7.0		+++	4.5		+++	5.0		+++	4.5		+++	4.5		+++	4.5
5	+	7.8	+	7.8		++	6.9		++	7.4		+++	5.4		+++	5.5		+++	4.5		+++	4.5		+++	4.5

## EXPERIMENTS WITH FATS AND OILS

We became interested in the effect of fats and oils as sources of growth promoting substances from the results obtained by Dr. Sherman of these laboratories, who found that fat-soluble A was apparently necessary for the growth of some of the organisms of the high acid group.

In all of our experiments the following basic medium was used:

Peptone (Difco).....	10 grams
Autolized yeast.....	10 grams
Distilled water.....	1,000 cc.
pH.....	7.4

Fifty cubic centimeters of this medium were placed in 100 cc. Erlenmeyer flasks and 0.5 cc. of the fat or oil to be studied was added before sterilization at 15 pounds for thirty minutes. This gave a concentration of 1 per cent of the oil in the medium. In the first experiments butter-fat and cod-liver oil were used. The butterfat was prepared from sweet cream churned into butter and washed with warm water several times. As a final process the fat was filtered through paper into a clean, dry beaker in which it was stored at about 5°C. during the course of these experiments. Using the same culture of streptococcus employed in the experiments with water-soluble B it was found that both butterfat and cod-liver oil stimulated the growth considerably during the first twenty-four hours. These experiments were repeated several times and the same results were obtained. While these results indicated that fat-soluble A might be responsible for the stimulation it was decided to try olive oil which is believed not to contain this vitamin. The same stimulation occurred however as with butterfat and cod-liver oil. This seemed to present evidence that fat-soluble A was not responsible for the stimulation.

While it is generally considered that olive oil does not contain fat-soluble A, the work of Drummond and Coward (1920) indicates that it may be present in small amounts. For this reason it was decided to use oil which could hardly be suspected of containing fat-soluble A and white mineral oil was therefore

selected. Much to our surprise the same stimulation of growth of the streptococcus occurred as with butterfat and cod-liver oil.

In these preliminary experiments there was no accurate measure of the degree of stimulation of growth, for it had been judged only by the appearance of the turbidity of the cultures after shaking. A more accurate measure was desired and the following method was adopted. Flasks containing 50 cc. of the yeast peptone medium with 1 per cent of a number of different oils<sup>2</sup> were inoculated with a loopful of a water suspension of a twenty-four-hour growth of the streptococcus on agar. After twenty-four hours' incubation at 37°C. the amount of growth was determined by plating on infusion agar. The results of these experiments are shown in table 3. It will be seen that the inoculated control peptone yeast medium without oil contained 170,000 bacteria per cubic centimeter after twenty-four hours of incubation. With sesame oil there was apparently little if any stimulation, while chaumogra oil seemed to be toxic. The rest of the oils and fats increased the count to a considerable degree. Some of the vegetable oils were considerably active, as for example okra-seed oil. This result is based on only one experiment, and in a repetition of the experiment a count was obtained with okra-seed oil similar to those observed with the other oils.

The most interesting feature of the results was the marked stimulation of growth by mineral oil, vaseline, and even solid paraffin. Microscopic examinations were made in some cases to prove that the plate count represented a difference in the actual number of cells in the cultures. Since we were working with a chain-forming streptococcus, the influence of the oil might have been to decrease chain formation, and therefore apparently increase the count. Microscopic tests showed, however, that the plate count represented the real difference in growth.

The results so far were obtained in media containing 1 per cent of the various oils but it was thought that this percentage could be lowered. To this end oils were selected from the three

<sup>2</sup> We are indebted to Doctor Jameison of the Bureau of Chemistry for many of the oils used in our experiments.

major groups (animal, vegetable, and mineral) and the amount of fat diminished to 0.2 per cent, 0.1 per cent, 0.02 per cent and 0.002 per cent. In table 4 the results are given. It will be seen that the counts tended to decrease with the lessened amount

TABLE 3  
*Effect of various fats and oils on the growth of a streptococcus*

FAT OR OIL (1 PER CENT USED)	REMARKS	BACTERIA PER CUBIC CENTIMETER AFTER TWENTY-FOUR HOURS
Control without oil.....		170,000
Sesame.....		400,000
Chaulmoogra.....		less than 100,000
Castor.....		1,100,000
Corn.....	Crude	2,600,000
Rape seed.....	Crude	3,500,000
Lumbang.....	Candle nuts	4,100,000
Coconut.....	Refined	6,000,000
Peanut.....	Crude	8,800,000
Peanut.....	Refined	11,000,000
Soy bean.....		10,400,000
Olive.....		9,700,000
Chia seed.....		10,400,000
Mustard seed.....		14,000,000
Okra seed.....		33,000,000
Linseed.....	Raw	5,600,000
Lard.....	Prime steamed	3,900,000
Lard.....	Open kettle rendered, direct heat	1,300,000
Lard.....	Prime steamed, refined	5,100,000
Butterfat.....	From sweet cream	14,100,000
Cod liver.....		8,100,000
Mineral.....	1 per cent added	5,700,000
Mineral.....	2 per cent added	5,100,000
Vaseline.....	1 per cent added	9,100,000
Vaseline.....	2 per cent added	9,700,000
Paraffine, solid.....	1 per cent added	5,900,000
Paraffine, solid.....	2 per cent added	11,100,000

of fat or oil but even small quantities caused a marked stimulation. In plating these oil media it was difficult to keep the 1 cc. sample drawn from the flask free from drops of oil and perhaps this is the explanation of some of the inconsistencies seen



in the tables. In making the dilutions of 0.02 per cent and 0.002 per cent of the fats and oils the following methods were used: To dilution blanks containing 100 cc. of 1 per cent peptone, 1 cc. of the oil was added and an emulsion obtained by thorough shaking. By adding 1 cc. and 0.1 cc. to our media we obtained the percentages of fat mentioned above. The main point to be observed however is that even down to 0.002 per cent of oil in the medium there was a stimulation of growth. This small

TABLE 4

*Effect of small amounts of butterfat, rape and mineral oils on the growth of a streptococcus*

CONCENTRATION	BUTTERFAT	RAPE OIL	MINERAL OIL
	<i>Bacteria per cc.</i>	<i>Bacteria per cc.</i>	<i>Bacteria per cc.</i>
Control without fat or oil.....	680,000	680,000	680,000
1.0.....	10,000,000	13,600,000	32,000,000
0.2.....	2,140,000	12,000,000	25,000,000
0.1.....	1,260,000	17,900,000	17,900,000
0.02.....	6,900,000	13,900,000	6,900,000
0.002.....	5,000,000	5,600,000	3,100,000
Extracts (?) from fats and oils.....	10,300,000	5,600,000	3,600,000

	<i>Bacteria per cc.</i>
Butter 1 per cent.....	{ first experiment.....430,000 second experiment.....0 in 1:10,000 dilution
Butterfat 1 per cent.....	{ first experiment.....11,000,000 second experiment.....14,000,000

amount of oil could hardly be distinguished on the surface of the media even on careful examination. At the same time other media were made in which the oils butterfat, rape and mineral or extracts from them, were added, in the following way: 100 cc. of water was shaken up with 5 cc. of the fat and oils for a period of three hours. The bottles were than set aside in the laboratory for several days after which time was a layer of oil at the surface of the water but the water itself was slightly cloudy. Two cubic centimeters of this emulsion, if it was an emulsion, since we dealt with pure oil and distilled water, were added to

the basic media and inoculated with the streptococcus. In table 4 experiment 3 we see a most astonishing stimulation of growth.

Another interesting phenomenon was observed which might well be mentioned at this point. In 1918, Mr. Johnson of these laboratories prepared in the field, some pasteurized-cream butter without salt and also separated some of the butterfat free from casein. These were sealed in sanitary cans and sent to Washington. Since then they have been in our incubators at temperatures averaging 30°C. These cans were opened recently and the fat found to be in a melted condition. Samples were taken of the supernatant fat from a butter can and also some of the oil from the can of butterfat free from casein. Media were made with these two fats using 1 per cent. The results which are also shown in table 4 are peculiar to say the least. The fat from the butter itself seems to be toxic but the stored fat free from casein stimulated bacterial growth the same as our fresh butterfat. One other point of interest is that the stimulating effects of fats and oils was not manifest to any degree in a plain peptone medium but was evident in the yeast peptone medium.

It was found by the addition of methylene blue that an anaerobic condition did not exist in the media with fats and oils. It cannot be assumed therefore that an anaerobic condition was responsible for the stimulation of the growth of the streptococcus.

The experiments with fats and oils show one thing definitely which is that very small amounts of these materials stimulate in a most remarkable manner the growth of a streptococcus growing in a yeast peptone medium. If our studies had been limited to butterfat and cod-liver oil strong evidence could have been presented as to the stimulation by fat-soluble A. But the stimulating effect of mineral oils and even solid paraffin change the aspect of the situation.

Our results as we see them permit of one of three possibilities, first, that the growth-promoting property of fats and oils is not due to the fat-soluble A, or second, that if fat-soluble A is responsible then it must be contained in mineral oil. The second

possibility suggests that it may be desirable to study further the vitamine content of mineral oil. Third, that the stimulation with fats and oils containing vitamine-A and that with mineral oils is not due to the same thing. Facts in our possession at present do not suggest which possibility is the correct one.

#### SUMMARY AND CONCLUSIONS

1. The results presented in this paper apply only to the growth of a culture of a pathogenic streptococcus and do not necessarily apply to bacteria in general.

2. Autolized yeast extract contained a growth-promoting substance or substances for the streptococcus studied. Water-soluble B did not, however, appear to be the significant substance.

3. Cabbage extract was found to promote growth, but a glucose solution containing the same amount of sugar as the cabbage extract showed a similar growth-promoting effect. It is evident that when extracts of plant or animal tissues are used the sugar content must be given consideration in connection with their growth-promoting properties.

4. Fats and oils, vegetable, animal and mineral even in very small amounts were found to stimulate the growth of the streptococcus. Either the growth-promoting property of fats and oils is not due to fat-soluble A, or this vitamine is present in mineral oils, or the stimulation is due to different causes in the case of the vitamine-containing fats and oils and the mineral oils.

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# SALT EFFECTS IN BACTERIAL GROWTH<sup>1</sup>

## II. THE GROWTH OF BACT. COLI IN RELATION TO H-ION CONCENTRATION

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It has been shown in a previous publication (Holm and Sherman, 1921) that sodium chloride and various other neutral salts in 0.20M concentration affected the rate of growth of Bact. coli. Using neutral salts with a common cation (sodium) but with various anions a marked difference was observed between the action of the various salts. The effect of the chlorides of sodium, potassium and ammonium seemed to be approximately the same while the calcium and iron salts tested retarded greatly or inhibited growth. These experiments were carried out at a pH of approximately 7.0 and with a salt concentration of 0.20M in 1 per cent<sup>1</sup> pepton.

In as much as we know that there are limiting pH values for bacterial growth, varying with different organisms, it would be of interest and value to know just to what extent this neutral salt action is affected by various H-ion concentrations. In the following experiments, as in our former communication, the rate of growth was determined by the time that expired between inoculation and the first sign of turbidity. The medium used was 1 per cent pepton to which had been added various amounts of salts, and the H-ion concentration adjusted by the use of concentrated HCl and NaOH solutions.

The effects of various concentrations of NaCl at various H-ion concentrations were first tried. The H-ion and salt concentra-

<sup>1</sup> Published with the permission of the Secretary of Agriculture.

tions, and the time for growth in each series, are shown in table 1. This table shows a decided accelerating effect upon growth with added NaCl in low concentrations. Although there was little difference between the effects of 0.10, 0.20 and 0.30M NaCl media upon growth, there seems to be an optimum effect at about 0.20M. The optimum H-ion concentration for growth either in controls or in pepton containing NaCl at various concentrations seems to be about the same, approximately pH 7.8. At optimum salt concentrations there is very little difference in the rate of growth over a wide range of H-ion concentration,

TABLE 1

*The rate of growth of Bact. coli in various concentrations of NaCl in 1 per cent pepton and at various H-ion concentrations*

NaCl CONCENTRATION	SERIES I		SERIES II		SERIES III		SERIES IV		SERIES V	
	pH	Hours	pH	Hours	pH	Hours	pH	Hours	pH	Hours
Control	5.3	36	6.3	10 $\frac{3}{4}$	7.0	7	7.7	6	8.3	7
0.05M	5.3	6 $\frac{3}{4}$	6.3	8 $\frac{1}{2}$	7.1	5 $\frac{3}{4}$	7.7	3 $\frac{3}{4}$	8.3	4
0.10M	5.3	4	6.4	4	7.1	4	7.8	3 $\frac{1}{2}$	8.3	3 $\frac{1}{2}$
0.20M	5.3	4	6.5	3 $\frac{1}{2}$	7.2	3 $\frac{1}{2}$	7.8	3 $\frac{1}{4}$	8.3	3 $\frac{1}{2}$
0.30M	5.3	4 $\frac{1}{2}$	6.5	3 $\frac{3}{4}$	7.3	3 $\frac{3}{4}$	7.9	3 $\frac{1}{4}$	8.3	3 $\frac{1}{2}$
0.40M	5.3	5 $\frac{1}{4}$	6.5	4 $\frac{1}{2}$	7.3	4	7.9	4	8.3	4

varying from 5.3 to 8.3 on the pH scale, while in the pepton solution alone the range is somewhat narrower. Beyond the range for optimum growth there seems to be a decided retardation for each small change of H-ion concentration. These results are brought out more clearly in figure 1 which shows a pronounced widening of the limiting pH values for growth with added NaCl, especially in optimum salt concentrations, and a retardation of growth for each small change in pH near the limiting values.

In order to ascertain if there was actually a shifting of the limits of growth, or merely a widening of the optimum range for growth, the effect of NaCl was tried at pH values representing the approximate limit of growth in the acid region. At a pH of 4.8 it was found that only rarely would *Bact. coli* grow in 1 per cent pepton at 37°C., but that it did grow quite readily in the

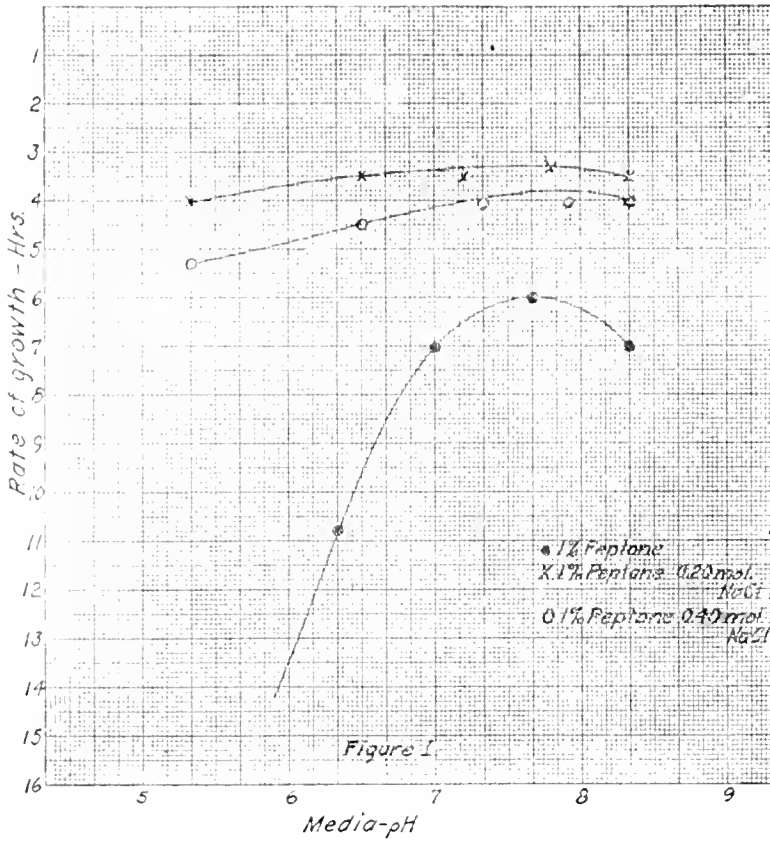


TABLE 2

The shifting of the limit of growth of *Bact. coli* at 37°C. in the acid region by the addition of NaCl

TEST NUMBER	TIME REQUIRED TO SHOW TURBIDITY AT A pH VALUE OF 4.8 IN	
	1 per cent pepton	1 per cent pepton 0.20 m NaCl
	<i>hours</i>	<i>hours</i>
1	No growth	18
2	No growth	22
3	No growth	18
4	No growth	18
5	26	21

same medium to which had been added NaCl to make a 0.20M solution. These media were adjusted colorimetrically, the end point being determined with methyl red, and it is of course recognized that there may be a slight error in the measurements. The significant fact is not whether the point established is exactly pH 4.8 but that the limiting H-ion zone of growth may be

TABLE 3

*The shifting of the limit of growth of Bact. alkaligenes at 37°C. in the acid region by the addition of NaCl*

MEDIUM	TEST NUMBER	TIME REQUIRED TO SHOW TURBIDITY AT pH VALUES OF		
		5.6	5.4	5.2
		hours	hours	hours
1 per cent pepton .....	1	90	No growth	No growth
1 per cent pepton.....	2	No growth	No growth	
1 per cent pepton 0.20M NaCl.....	1	24	24	120
1 per cent pepton 0.20M NaCl.....	2	24	24	

TABLE 4

*The effect of NaCl and Na citrate upon Bact. coli at various H-ion concentrations*

MEDIA	TIME REQUIRED TO SHOW TURBIDITY AT pH VALUE OF					
	4.8	5.2	6.2	7.6	8.2	9.2
	hours	hours	hours	hours	hours	hours
1 per cent pepton.....	No growth	16	6	5½	8	32
1 per cent pepton 0.20M NaCl..	18	5½	4	3½	3½	14
1 per cent pepton 0.20M Na citrate.....	No growth	26	8½	7½	22	No growth

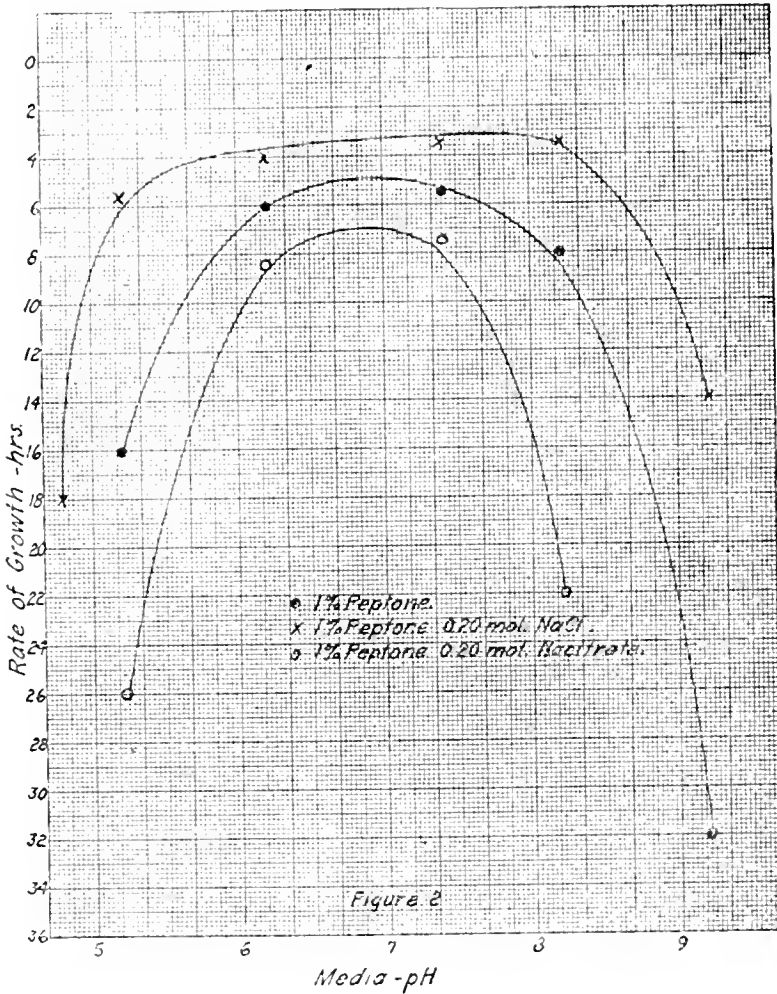
modified by the addition of NaCl to the medium. The results of five different tests made under these conditions are recorded in table 2.

Although the widening effect upon the pH limit of growth is not general for all bacteria which we have tried, we have found the effect upon *Bact. alkaligenes* to be even more pronounced than the effect upon *Bact. coli*. This is shown in table 3.

Tables 2 and 3 show that there is actually an extension of the zone in which *Bact. coli* and *Bact. alkaligenes* will grow in the



acid region by the addition of 0.20M NaCl. It is possible that this effect might be increased by using a more dilute salt solution, and perhaps, in the same way, this effect might be produced



with organisms which thus far have failed to show any such modification in their limits of growth.

The same results which we have noted with NaCl may be produced with other neutral salts, but the degree of the widening

effect varies with the nature of the salt. On the other hand, a salt (e.g., Na citrate) which lowers the rate of growth also narrows the limits of H-ion concentration at which *Bact. coli* will grow. Table 4 shows the results obtained with NaCl and Na citrate, as compared with pepton alone, at different H-ion concentrations. These results are shown to better advantage in figure 2 which brings out clearly the widening effect on the limits of growth with NaCl, while Na citrate shows a decided narrowing of the range.

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A NOTE ON THE MORPHOLOGY OF BACTERIA  
SYMBIOTIC IN THE TISSUES OF HIGHER  
ORGANISMS

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In connection with a study of the cytoplasmic relationships of root-nodule bacteria in the common white clover, the author found a stage in the morphogenesis of *Bacillus radicum* that, apparently, is not well known. A careful search was made in textbooks of bacteriology for a description or mention of this form, but in vain. Through the courtesy of Dr. F. Löhnis of the United States Department of Agriculture, I have been able to find references to similar forms. Doctor Löhnis (1921) in his exhaustive review of the literature on the life cycles of the bacteria describes and illustrates round forms of *Bacillus radicum* which appear to be similar to the spherical forms that I found. Löhnis and Smith (1916) were the first investigators to observe the spherical forms. More recently Bewley and Hutchinson (1920) have observed, apparently, the same form in cultural conditions and call this stage in the morphogenesis of the organism the "swarmer" stage.

In relation to the spherical forms that have been described by Löhnis (gonidia, regenerative units) it remains to be decided whether the spherical forms that I have called "senile" are derived from the branched forms (bacteroids) or from the symplasm. The relationship and character of these spherical forms in the root nodule is decidedly interesting and I believe that a study of these forms in sections of the root nodule may be valuable in the interpretation of their nature. It appears to the author that these spherical forms are fragile and are generally destroyed in the ordinary bacteriological technique.

When my specimens were first examined with a low magnification of the microscope I was impressed by what appeared to be three distinct regions or areas in the root nodule. On closer examination with oil immersion lens, the three areas were found to contain three distinct forms of organisms. Each form was more or less limited to a single area. In the part of the nodule next to the plant root, the nodule cells contained no other than the spherical forms. I interpret this part of the nodule to be the older part and on the basis of this interpretation have called the spherical cells "senile" forms. The "bacteroid" forms of the bacillus were likewise limited, almost entirely, to the central portion of the nodule. In what I interpret to be the younger part of the nodule the bacilli were all of the small variety and not nearly so numerous as the other forms.

My interest in these forms is not "bacteriological" and I have no desire to pursue the investigation any further, at least for the present. However, it does occur to me that the technique that I have used may be valuable in bacteriological research. In a recent publication (Wallin, 1922a), I submitted evidence that mitochondrial methods are not specific, but will also stain bacteria. It has since occurred to me, particularly in connection with my study of root-nodule bacteria, that the mitochondrial technique may, at least in some cases, be superior to the usual bacteriological methods, particularly when dealing with symbiotic bacteria. I have tested a number of mitochondrial methods on various kinds of bacteriological material: sputum smears, pus smears and sections, tissue smears, bacterial smears, etc. In the majority of instances the differentiation between bacterium and tissue has been decidedly sharp.

In staining the root nodules, I have used only one method. This consisted in fixation of the entire nodule in a modification of Flemming's fixative: 4 cc. 2 per cent aqueous solution of osmic acid and 6 cc. 1 per cent aqueous solution of chromic acid. (Fix from four to twenty-four hours.) After washing, dehydration, clearing, and embedding in hard paraffin (58°), sections were cut 3 micra in thickness. The sections were mounted on slides and stained by Bensley's (1911) anilin fuchsin-methyl green method:

The staining solutions are:

1. Altmann's acid fuchsin anilin solution:
 

Acid fuchsin.....	20 grams
Anilin water.....	100 cc.
2. 1 per cent solution of methyl green

The sections after being prepared for the staining process by treatment with permanganate of potassium followed by oxalic acid, are stained for five minutes in the acid fuchsin solution which has been previously warmed to 60°C. Next they are thoroughly washed in distilled water, and dipped for an instant into the solution of methyl green, then washed, rapidly dehydrated in absolute alcohol (alcohol of intermediate strength must be avoided) cleared in toluol, and mounted in balsam.

The author has found that the permanganate and oxalic acid treatment may be omitted if the fixation has not been carried too far. However, if the staining differentiation is not sharp the sections may be treated for a minute or so in 1 per cent permanganate of potassium and followed by a similar treatment in 5 per cent oxalic acid. I have also found a great variation in the quality of various brands of methyl green. I have only been able to get good results with Grübler's methyl green. This method, when carried out successfully, gives excellent differentiation between bacteria and tissues.

There are several mitochondrial methods in use. Benda's crystal violet method, perhaps, gives the sharpest differentiation of all these methods. This is a very long and tedious method, but in some cases the results appear to justify the longer procedure. Other methods are: Altmann's anilin fuchsin picric acid method, Schridde's modification of Altmann's method, Regaud's method, the copper and iron hemotoxylin methods, etc. I have stained bacteria by all these methods with good results.

The mitochondrial staining methods were devised to fix and stain delicate bodies (mitochondria) in the cytoplasm which are not visible after ordinary histological technique. It appears that some symbiotic bacteria, particularly those that have an intracellular relationship, are just as fragile and delicate as mito-

chondria. The author believes that his results on the root-nodule bacteria were due to this technique. For illustrations of the three forms of *Bacillus radiceicola*, the reader is referred to an article by the author (Wallin, 1922b).

Murray (1919) has recently recommended the use of mitochondrial technique in certain cases where the Gram stain, for example, does not give differential results.

The author also wishes to call attention to the action of janus green when applied to bacteria. All bacteria, apparently, are not stained by janus green, but certain strains are and, apparently, only under certain conditions. This is a vital stain and it is possible that it may be found useful in the study of certain bacteria. The reader is referred to Cowdry (1918) for directions and information regarding the proper brand of janus green.

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# OBSERVATIONS ON THE PROPERTIES OF BACTERIOLYSANTS (D'HERELLE'S PHENOMENON, BACTERIOPHAGE, BACTERIOLYTIC AGENT, ETC.)

## PART I<sup>1</sup>

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### INTRODUCTION

Twort, d'Herelle and others (Davison, 1922a) have observed that Berkfeld filtrates of stool and other cultures would kill and dissolve young cultures of dysentery and other organisms. The addition of a portion of one of these dissolved cultures, to a new culture would cause it in turn to dissolve. Thus the lytic principle could be transferred from generation to generation. These bacteriolytic filtrates or their subsequent generations are called, interchangeably, bacteriolysants, bacteriophages and bacteriolytic agents. The process by which these filtrates dissolve dysentery and other organisms is known as d'Herelle's phenomenon or bacteriophagy.

It has been shown (Davison, 1922a) that agar subcultures of organisms which had been attacked by bacteriophages, contained two types of colonies. One was regular and round, resembling a typical dysentery colony. It was not readily attacked by the bacteriolytic agent. This is the "resistant" type. The other was irregular in outline and is best described as "moth eaten." These colonies were easily dissolved by bacteriophages and in addition had the property of dissolving other cultures to which they were added. This is the "sensitive"

<sup>1</sup> Presented at the twenty-third annual meeting, Society of American Bacteriologists, December 29, 1921. From the Department of Pediatrics, of the Johns Hopkins University and the Harriet Lane Home of the Johns Hopkins Hospital, Baltimore, Maryland.

or "lysogenic" strain. A "normal" strain is one that has never been in contact with a bacteriophage.

#### STOOL CULTURE FILTRATES

I have obtained stool culture filtrates by inoculating a loopful of feces into 100 cc. of 1 per cent peptone water at pH 8. After incubation at 37°C. from twelve hours to five days, these cultures were filtered through a Mandler no. 6 candle. The filtrates were then incubated at 37°C. for forty-eight hours to prove their sterility. Filtrates were made from the stools of ten infants (table 1). Seven of these children suffered from bacillary dysentery (Flexner).<sup>2</sup> Two of these had *Bact. dysenteriae* in their stools at the time the filtrates were obtained. One of these two patients died. Two infants suffered from acute intestinal ingestion and one was a case of Otitis Media and feeding regulation. A bacteriophage obtained from the stool of an adult convalescent typhoid patient<sup>3</sup> has been used as a comparison. It was active against several normal strains of Flexner bacilli (table 1).

#### METHODS OF TESTING THE BACTERIOLYTIC ACTIVITY OF BACTERIOLYSANTS

The bacteriolytic activity of these various filtrates was tested as follows: six tubes each containing 2 cc. of sterile 1 per cent peptone water (pH 8.0) were each inoculated with one drop of a fluid culture of *Bact. dysenteriae* (Flexner). After two to twelve hours' incubation at 37°C., 0.5 cc. of the filtrate to be tested was added to the first tube (making a dilution of 1:5), 0.25 cc. to the second tube (a dilution of 1:9), and 0.1 cc. to the third tube (a dilution of 1:21). Sterile peptone water in amounts of 0.5 cc., 0.25 cc. and 0.1 cc. was added to the fourth, fifth and sixth tubes which served as controls. These six tubes were

<sup>2</sup> By Flexner bacilli, I refer to the whole group of mannitol fermenting dysentery bacilli. By Flexner V, W, X, Y or Z, I refer to the English serological divisions of this group (Davison, 1922).

<sup>3</sup> This bacteriophage was given me by Miss Ann Kuttner of the Department of Bacteriology, Columbia University, to whom my thanks are due.



TABLE 1  
*Bacteriolytic activity of stool culture filtrates*

Name of patient	SOURCE OF BACTERIOLYSANT		NUMBER OF DIFFERENT CULTURES WITH WHICH BACTERIOLYSANTS WERE TESTED	NUMBER OF LYSIS TESTS MADE WITH ORIGINAL STOOL FILTRATE OR WITH SUBSEQUENT GENERATIONS OF IT	NUMBER OF SUBSEQUENT GENERATIONS TO WHICH ORIGINAL STOOL FILTRATE WAS CARRIED	LYTIC ACTIVITY OF SUBSEQUENT GENERATIONS COMPARED WITH THAT OF ORIGINAL STOOL FILTRATE	ACTIVITY OF BACTERIOLYSANT OBTAINED FROM PATIENT AGAINST DYSENTERY BACILLI ISOLATED FROM PATIENT AS COMPARED TO ITS ACTIVITY AGAINST OTHER STRAINS OF FLEXNER BACILLI
	Diagnosis of patient	Outcome of case					
J. B. (infant) . . . .	Dysentery (Flexner)	Recovery	9	17	3	Same	Greater
E. B. (infant) . . . .	Dysentery (Flexner)	Recovery	11	19	3	Greater	Same
T. B. (infant) . . . .	Dysentery (Flexner)	Recovery	16	31	2	Same	Not tested
M. C. (infant) . . . .	Dysentery (Flexner)	Recovery	11	11	2	Not tested	Not tested
I. J. (infant) . . . .	Dysentery (Flexner)	Recovery	8	9	2	Less	Less
D. R. (infant) . . . .	Dysentery (Flexner)	Recovery	6	11	0	Same	Not tested
J. S. (infant) . . . .	Dysentery (Flexner)	Death	5	10	1	Same	Less
A. L. (infant) . . . .	Acute intestinal indigestion	Recovery	9	12	0	Greater	Less
E. A. (infant) . . . .	Acute intestinal indigestion	Recovery	1	1	0		
T. T. (infant) . . . .	Otitis media and regulation of feeding	Recovery	20	75	4	Greater	
Adult . . . . .	Typhoid fever	?	10	29	8	Same	Not tested

than incubated at 37°C. and the degree of lysis was noted macroscopically at various intervals for seventy-two hours.

Inasmuch as it had been shown (Davison, 1922a) that agar subcultures of organisms which have been attacked by bacteriolysants contain "sensitive," "moth eaten" colonies, the presence of such colonies in subcultures was used as confirmatory evidence of the occurrence of lysis. However, subcultures of some bacterial suspensions which had apparently been lysed by a filtrate contained nothing but regular colonies, so that their absence does not necessarily mean that lysis had not occurred.

Frequently when normal dysentery bacilli, which had been incubated with a bacteriolysant, were subcultured in peptone water these subcultures failed to grow, although agar subcultures made at the same time, contained a number of "sensitive" and regular colonies. It is possible that there was sufficient bacteriolysant carried over in the subculture loop to inhibit growth in a fluid medium while on agar, this small amount was lost in streaking across the plate.

The amount of bacteriolysant added to a culture of dysentery bacilli, apparently does not have a quantitative effect on the percentage of "sensitive" colonies which are found in agar subcultures of these bacilli, i.e., subcultures of peptone water cultures of dysentery bacilli some of which had been incubated with 0.5 cc. and others with 0.1 cc. of a bacteriolysant frequently had the same percentage of "sensitive" colonies.

In several instances I have confirmed these determination of lysis by counting the organisms (plate counts) before and after the action of a bacteriolysant and have found the reduction of viable organisms to be proportional to the degree of lysis, i.e., a culture which contained 100,000,000 Flexner bacilli per cubic centimeter at the commencement of the experiment, and which after twenty-four hours' contact with a filtrate was apparently completely lysed, contained two viable organisms per cubic centimeter (table 2). It is, of course, impossible to state whether this reduction in the number of viable organisms is altogether the result of the bactericidal action of the bacteriolysant or whether inhibition does not also play a part. Several cultures which

had been attacked in varying degrees by filtrates were centrifuged and the sediment stained. The amount of cellular debris was variable but the number of morphologically typical dysentery

TABLE 2

*Comparison of bacteriolytic activity of bacteriolysants with the bacterial counts of the culture before and after the action of bacteriolysants*

0.5 CC. OF EACH OF THE FOLLOWING BACTERIOLYSANTS WERE ADDED TO 2 CC. OF AN EIGHT HOUR PEPTONE WATER CULTURE OF FLEXNER Y, AND THEN INCUBATED TOGETHER AT 37°C. FOR TWENTY-FOUR HOURS	NUMBER OF ORGANISMS PER CUBIC CENTIMETER IN THE EIGHT HOUR PEPTONE WATER CULTURE OF FLEXNER Y, BEFORE BACTERIOLYSANT WAS ADDED	DEGREE OF LYSIS AFTER CULTURE AND BACTERIOLYSANT WERE INCUBATED TOGETHER AT 37°C. FOR TWENTY-FOUR HOURS	NUMBER OF VIABLE ORGANISMS PER CUBIC CENTIMETER AFTER CULTURE AND BACTERIOLYSANT WERE INCUBATED TOGETHER AT 37°C. FOR TWENTY-FOUR HOURS
F 24(3)A.....	100,000,000 +	+	264
F 49(2).....	100,000,000 +	+	41
F 43(1).....	100,000,000 +	++	2
F 44.....	100,000,000 +	+	130
(Control) 1 per cent sterile peptone water.....	100,000,000 +	0	100,000,000 +

TABLE 3

*Comparison of the degree of bacteriolysis with appearance of the stained sediment*

0.5 CC. OF EACH OF THE FOLLOWING BACTERIOLYSANTS WERE ADDED TO 2 CC. OF AN EIGHT HOUR PEPTONE WATER CULTURE OF FLEXNER Y, AND THEN INCUBATED TOGETHER AT 37°C. FOR SEVENTY-TWO HOURS	DEGREE OF LYSIS AFTER THE CULTURE AND BACTERIOLYSANT WERE INCUBATED TOGETHER AT 37°C. FOR SEVENTY-TWO HOURS	APPEARANCE OF THE STAINED SEDIMENT (GRAM'S STAIN) AFTER THE CULTURE AND BACTERIOLYSANT HAD BEEN INCUBATED TOGETHER AT 37°C. FOR SEVENTY-TWO HOURS AND THEN CENTRIFUGED	
		Amorphous cellular debris	Morphologically typical dysentery bacilli
F 24 (3) A.....	+++	±	±
F 49 (2).....	++	+++	±
F 34 (1).....	++	++	±
F 43 (1).....	+	±	±
F 31.....	+	++	++
F 45.....	+	+++	++
F 48.....	+	+++	++
F 44.....	±	+	+++
(Control) 1 per cent sterile peptone water.....	0	0	+++

bacilli was inversely proportional to the amount of lysis that had occurred (table 3).

The bacteriolytic activity of these filtrates was also tested in several instances by dropping a small portion of the filtrate on the surface of an agar plate which had been heavily inoculated five to twenty-four hours previously with Flexner bacilli. These plates were then incubated for twenty-four hours and the presence or absence of macroscopic lysis in the area bathed by the filtrate was noted. The presence of "moth eaten" colonies in subcultures of these areas confirmed the occurrence of lysis.

#### ORGANISMS ATTACKED BY STOOL BACTERIOLYSANTS

Five stock laboratory Flexner strains, twenty freshly isolated Flexner strains, one stock laboratory Shiga strain and one stock laboratory typhoid strain were all lysed by one or more of sixty-eight bacteriolysants (either original stool filtrates or their subsequent generations) obtained from eleven patients (table 1).

#### VARIATIONS IN THE BACTERIOLYTIC ACTIVITY OF STOOL BACTERIOLYSANTS

All of the sixty-eight bacteriolysants, some of which were tested against as many as eleven among this total of twenty-seven strains were active against one or more strains. Some filtrates attacked several strains, others only one. Among the two hundred and twenty-five lysis tests performed with these sixty-eight filtrates and twenty-seven strains, twenty-five tests were negative.

Among fifty filtrates which were tested against two to eleven different strains, fifteen (or 30 per cent) had the same titre of lysis against all of the organisms against which they were tested, i.e., filtrate no. 31 (4) when diluted 1:21 lysed all seven of the strains of the Flexner bacillus against which it was tested. Thirty-five filtrates (or 70 per cent) produced lysis in different dilutions, i.e., filtrate no. 33 (1) lysed six strains at a dilution of 1:21 and one strain at 1:5. The bacteriolytic titre of eight filtrates was tested against four dysentery cultures obtained by fishing four separate colonies from the same plate of a stool culture. Six of these filtrates lysed all four cultures at a dilution of 1:21 while the seventh filtrate lysed one culture at 1:21, two cultures

at 1:9 and one at 1:5 and the eighth filtrate attacked two cultures at 1:21 and two at 1:5. It is thus obvious that cultures differ in "lysability" just as they vary in "agglutinability." One Flexner strain was tested with forty-eight different bacteriolyants, and was lysed by thirty-nine at a dilution of 1:21, by three at a dilution of 1:9, by five at a dilution in 1:5 and was not lysed at all by one.

The bacteriolytic power of subsequent generations of a stool filtrate, obtained by filtering a dysentery culture which had been lysed by being in contact for twenty-four hours or more with a stool filtrate, in some instances was the same, in others greater and in others less than that of the original filtrate. The type of dysentery bacillus to which the original filtrate was added in the preparation of these subsequent generations, apparently did not affect the titre of the succeeding generation of filtrate. Among four filtrates that were carried through three to six generations and were always tested against the same organism, two had the same titres of lysis after each transfer and two had different titres, i.e., after one "passage" lytic action might be increased and after another "passage" it might be decreased. Among three filtrates that were carried three to ten generations and were always tested against two or more organisms, the titre of lysis in each generation remained the same for one organism and was different (increased or decreased) for the others.

Twelve first to sixth generation filtrates, the originals of which were obtained from the stools of four dysentery patients, were tested against the dysentery bacillus isolated from those patients' stools as well as against other strains of *Bact. dysenteriae*. Four of these filtrates were more active against their own patient's organism than the other strains, four were less active and four were equally active.

The bacteriolytic power of filtrates that had become contaminated with stool or air organisms and were then refiltered, was sometimes the same, sometimes greater and sometimes less than that of the original filtrate. Among nine contaminated filtrates that were refiltered three had increased lytic power, three had decreased activity and in three the activity was unchanged.

Among thirteen filtrates heated immediately after filtration to 60 to 67°C. for forty-five to sixty minutes, the titre of lysis was reduced in five, apparently increased in two and unchanged in six. The changes were however so slight as to be within the range of experimental error, so that it is probable that the bacteriolytic power of a filtrate is unaffected by these temperatures.

The degree of lysis is also dependent upon the concentration of the bacteriolytic filtrate, lysis frequently occurring at a dilution of 1:5 and being absent at 1:9, or 1:21.

LOSS OF BACTERIOLYTIC ACTIVITY WHEN A BACTERIOLYSANT IS  
SUCCESSIVELY SUBCULTURED IN STERILE PEPTONE WATER  
OR IN A CULTURE KILLED BY HEAT

A bacteriolysant was added to a flask of sterile peptone water incubated twenty-four hours at 37°C. and then filtered. A portion of this filtrate was then added to another flask of sterile peptone water, incubated twenty-four hours at 37°C. and then filtered. A portion of this filtrate was then added to another flask of sterile peptone water, incubated twenty-four hours and then filtered. This was repeated for six such "passages." The bacteriolytic activity which was marked in the original bacteriolysant became progressively weaker and disappeared after the fifth passage, i.e., when the original bacteriolysant was diluted 1:7776. Although undemonstrable in the fourth "passage" when the original bacteriolysant was diluted 1:1296, yet subcultures of the dysentery bacilli which had been incubated with this filtrate contained a few "sensitive" colonies so that a slight amount of lytic activity was probably present at that dilution (table 4).

The effect of filtration on the bacteriolytic activity of these preparations was also studied, i.e., after the bacteriolysant had been incubated with sterile peptone water, the bacteriolytic activity of a portion of it was tested. The remainder was then filtered and the bacteriolytic activity of this filtrate tested. The titre of lysis of the unfiltered and the filtered portions was the same.

TABLE 4

*Loss of bacteriolytic activity when bacteriolysant is successively "subcultured" in sterile peptone water*

FILTRATE USED	BACTERIOLYTIC ACTIVITY OF FILTRATE AGAINST CULTURES OF NORMAL FLEXNER DYSENTERY BACILLI	PRESENCE OF "SENSITIVE" COLONIES IN AGAR SUBCULTURES OF DYSENTERY BACILLI AFTER THEY HAD BEEN INCUBATED WITH THE FILTRATE FOR TWENTY-FOUR HOURS AT 37°C.	DILUTION OF ORIGINAL BACTERIOLYSANT IN PEPTONE WATER FILTRATE
<i>Original bacteriolysant</i>	++	Not sub-cultured	
<i>First "passage" in sterile peptone water, i.e., 20 cc. of original bacteriolysant added to 100 cc. sterile peptone water, incubated twenty-four hours at 37°C. and then filtered</i>	±	++	1:6
<i>Second "passage" in sterile peptone water, i.e., 20 cc. of 1st "passage" filtrate added to 100 cc. sterile peptone water, incubated twenty-four hours at 37°C. and then filtered</i>	Doubtful	+	1:36
<i>Third "passage" in sterile peptone water, i.e., 20 cc. of second "passage" filtrate added to 100 cc. sterile peptone water, incubated twenty-four hours at 37°C. and then filtered</i>	Very doubtful	+	1:216
<i>Fourth "passage" in sterile peptone water, i.e., 20 cc. of third "passage" filtrate added to 100 cc. sterile peptone incubated twenty-four hours at 37°C. and then filtered</i>	0	±	1:1296
<i>Fifth "passage" in sterile peptone water, i.e., 20 cc. of fourth "passage" filtrate added to 100 cc. sterile peptone water, incubated twenty-four hours at 37°C. and then filtered</i>	0	0	1:7776
<i>Sixth "passage" in sterile peptone water, i.e., 20 cc. of fifth "passage" filtrate added to 100 cc. sterile peptone water incubated twenty four hours at 37°C. and then filtered</i>	0	0	1:46,656

An eighteen-hour peptone water culture of normal Flexner bacilli was killed by being heated to 60 to 65°C. for one hour (table 5). A bacteriolysant was then added to this dead culture and the preparation incubated twenty-hour hours at 37°C. and then filtered. This filtrate would not produce macroscopic lysis, but subcultures of the dysentery bacilli to which the filtrate was added contained a few "sensitive" colonies so that a certain amount of lytic activity was probably present. However this amount was less than that contained in a bacteriolysant after "passage" through sterile peptone water and almost negligible when compared with the bacteriolytic activity of a bacteriolysant after "passage" through a live culture of Flexner bacilli (table 5). The slight amount of bacteriolytic activity that this filtrate contained is probably due to the fact that there was sufficient of the original bacteriolysant present to cause the production of "sensitive" colonies. The fact that "passage" through a dead culture reduced the bacteriolytic activity of the bacteriolysant more than "passage" through the same amount of sterile peptone water suggests that the dead bacteria may have adsorbed the lytic principle in much the same way that kaolin adsorbs enzymes.

THE EFFECT OF THE REACTION OF THE MEDIA IN WHICH DYSENTERY  
BACILLI WERE GROWN, ON THE DEGREE OF LYSIS PRO-  
DUCED IN A CULTURE BY A BACTERIOLYSANT

Flasks of 1 per cent peptone water, to which phenol-sulphone-phthalein was added, were adjusted to each of the following reactions: pH 6.0, 6.6, 7.1, 7.4, 7.7, 8.0 and 8.2. Two cubic centimeters of the media at each reaction were placed in several tubes and these were sterilized in the autoclave. These tubes were then inoculated with Flexner dysentery bacilli. After four to twenty-four hours' incubation, bacteriolysants were added and the degree of lysis produced was noted. As may be seen in table 6, cultures whose initial pH was 8.0 and 8.2 were lysed somewhat more completely than cultures whose reactions were from pH 6.0 to 7.7.



TABLE 5

*Comparison of effect on bacteriolytic activity of a bacteriolysant of "subculturing" it in a dead culture of normal Flexner bacilli, in a live culture of normal Flexner bacilli and in sterile peptone water*

FILTRATE USED	BACTERIOLYTIC ACTIVITY OF FILTRATE AGAINST CULTURES OF NORMAL FLEXNER DYSENTERY BACILLI	PRESENCE OF "SENSITIVE" COLONIES IN AGAR SUBCULTURES OF DYSENTERY BACILLI AFTER THEY HAD BEEN INCUBATED WITH THE FILTRATE FOR TWENTY-FOUR HOURS AT 37°C.	DILUTION OF THE ORIGINAL BACTERIOLYSANT IN "PASSAGE" FILTRATE
Original bacteriolysant	++	Not subcultured	
<i>Filtrate after "passage" in dead culture of Flexner dysentery bacilli, i.e., 20 cc. of original bacteriolysant added to 100 cc. of dead eighteen-hour peptone water culture of normal Flexner bacilli (killed by being heated to 60 to 65°C. for one hour), incubated twenty-four hours at 37°C. and then filtered</i>	0	+	1:6
<i>Filtrate after "passage" in live culture of Flexner dysentery bacilli, i.e., 20 cc. of original bacteriolysant added to 100 cc. of an eighteen-hour peptone water culture of normal Flexner bacilli, incubated twenty-four hours at 37°C. and then filtered</i>	+++	++	1:6
<i>Filtrate after "passage" in sterile peptone water, i.e., 20 cc. of original bacteriolysant added to 100 cc. of sterile peptone water, incubated twenty-four hours at 37°C. and then filtered</i>	±	++	1:6

COMPARISON OF BACTERIOLYTIC ACTIVITY OF BACTERIOLYSANT  
AGAINST SALINE AND PEPTONE WATER SUSPENSIONS  
OF NORMAL DYSENTERY BACILLI

Bacteriolysants were equally active against normal dysentery bacilli suspended in 0.9 per cent saline and 1 per cent peptone water provided the reactions of the two suspensions were the same (table 7). It is possible that earlier reports (Davison, 1922) of the failure of bacteriophages to lyse saline suspensions of dysentery bacilli may be explained by the fact that the reaction of the saline used, was too far from the optimum.

TABLE 6

*The effect of the reaction of the media in which dysentery bacilli were grown, on the degree of lysis produced in a culture by a bacteriolysant*

BACTERIOLY- SANT USED	FLEXNER DYSENTERY CULTURE USED IN LYSIS TESTS	INITIAL pH OF THE DYSENTERY CUL- TURE USED IN LYSIS TESTS						
		6.0	6.6	7.1	7.4	7.7	8.0	8.2
F 34(3)B ...	Four-hour peptone water culture of Flexner Y (Hiss and Russell)	±	+	+	+	+	+	++
F 42(2).....	Four hour peptone water culture of Flexner 120 (isolated from patient in H.L.H.)	±	±	±	±	±	+	+
F 48(3).....	Twenty-four hour peptone water culture of Flexner 90 (isolated from patient in H.L.H.)	+	+	+	+	+	+	++

“RESISTANCE” OF OLD PEPTONE WATER CULTURES TO  
BACTERIOLYSANTS

As shown in table 8, bacteriolysants had no bacteriolytic activity against a one hundred and thirty-day-old peptone water culture of normal Flexner Y bacilli. There are at least four explanations for this failure, i.e., (1) that the reaction of the culture was too far from the optimum, (2) that the culture contained so many organisms that the lytic principle was adsorbed by the bacterial bodies, (3) that the organisms were all “resistant” and (4) that the culture was dead. However, its reaction was pH 8.0 which is at or near the optimum. The concentra-

tion factor did not appear to be essential for when the culture was diluted with saline to the same opacity as a three hour culture it was not lysed. That the organisms were not all dead and "resistant" was demonstrated by the fact that sub-cultures of this old culture grew and also contained a few "irregular" colonies. The fact that the culture when diluted with 1 per cent peptone water was lysed by the bacteriolysant may be explained by the fact that the organisms grew in the peptone

TABLE 7

*Comparison of bacteriolytic activity of bacteriolysant against saline and peptone water suspensions of agar cultures of normal dysentery bacilli (Flexner Y, Hiss and Russell)*

BACTERIOLYSANT USED	BACTERIAL SUSPENSION USED TO TEST BACTERIOLYTIC ACTIVITY	BACTERIOLYTIC ACTIVITY OF BACTERIOLYSANT AGAINST THE BACTERIAL SUSPENSION
F 31(4).....	Growth from seven-hour agar culture of normal Flexner bacilli suspended in 0.9 per cent saline at pH 8.0	+
F 34(2)B.....	Growth from seven-hour agar culture of normal Flexner bacilli suspended in 0.9 per cent saline at pH 8.0	+
F 31(4).....	Growth from seven-hour agar culture of normal Flexner bacilli suspended in 1 per cent peptone water at pH 8.0	+
F 34(2)B.....	Growth from seven-hour agar culture of normal Flexner bacilli suspended in 1 per cent peptone water at pH 8.0	+

water which was added, and it was then a young culture that was being attacked. The most probable explanation for the greater bacteriolytic activity of bacteriolysants when tested against young cultures, is that many of the organisms in any culture older than twenty-four hours, are dead and act like kaolin in enzymatic phenomena and adsorb the bacteriolytic principle.

EFFECT OF SODIUM HYDROXIDE UPON A BACTERIOLYSANT

One cubic centimeter of normal sodium hydroxide was added to 4 cc. of a bacteriolysant and after eighteen hours incubation

at 37°C. the reaction was adjusted to pH 8.0 with normal hydrochloric acid (0.82 cc.). The bacteriolysant after this treatment had no bacteriolytic power, i.e., sodium hydroxide in a

TABLE 8  
*Bacteriolytic activity of bacteriolysants against old peptone water cultures of normal dysentery bacilli (Flexner Y, Hiss and Russell)*

BACTERIOLYSANT USED	CULTURE USED TO TEST BACTERIOLYTIC ACTIVITY	BACTERIOLYTIC ACTIVITY OF BACTERIOLYSANT AGAINST THE CULTURE TESTED
F 43(5).....	One hundred and thirty-day peptone water culture of Flexner Y (undiluted)	0
F 34(3)B.....	One hundred and thirty-day peptone water culture of Flexner Y (undiluted)	0
F 43(5).....	One hundred and thirty-day peptone water culture of Flexner Y (diluted ten times with 0.9 per cent saline) (to same opacity as a three-hour culture)	0
F 34(3)B.....	One hundred and thirty-day peptone water culture of Flexner Y (diluted ten times with 0.9 per cent saline) (to same opacity as a three-hour culture)	0
F 43(5).....	One hundred and thirty-day peptone water culture of Flexner Y (diluted ten times with 1 per cent peptone water) (to same opacity as a three-hour culture)	±
F 34(3)B.....	One hundred and thirty-day peptone water culture of Flexner Y (diluted ten times with 1 per cent peptone water) (to same opacity as a three-hour culture)	±
F 43(5).....	Three-hour peptone water culture of Flexner Y	++
F 34(3)B.....	Three-hour peptone water culture of Flexner Y	++

strength of N/5 destroyed the lytic principle, normal sodium hydroxide killed dysentery bacilli and also lysed the organisms. Twenty per cent hydrochloric acid killed the culture but did not lyse it (table 9). The organisms treated with hydrochloric acid did not stain well, however.

TABLE 9  
*Effect of sodium hydroxide upon a bacteriolysant*

BACTERIOLYSANT OR CHEMICAL USED	BACTERIOLYTIC ACTIVITY OF BACTERIOLYSANT OR CHEMICAL AGAINST CULTURES OF NORMAL FLEXNER BACILLI	PRESENCE OF "SENSITIVE" COLONIES IN AGAR SUBCULTURES OF DYSENTERY BACILLI AFTER THEY HAD BEEN INCUBATED WITH THE BACTERIOLYSANT OR CHEMICAL FOR TWENTY-FOUR HOURS AT 37°C.
F 34(3)B, after incubation for eighteen hours with N/5 NaOH and then adjusted to pH 8.0 with N/1 HCl	0	Not subcultured
F 34(3)B (without NaOH or HCl)	+	+
N/1 NaOH.....	+	No growth on subculture, i.e., culture killed
20 per cent HCl.....	0	No growth on subculture, i.e., culture killed

RESULTS OF INOCULATIONS OF BACTERIOLYTIC FILTRATES INTO ANIMALS

Four doses of 5 to 15 cc. of a bacteriolytic filtrate at intervals of six to seventy-five days were injected intravenously and subcutaneously into a rabbit. The animal showed no ill effects. Its serum after the fourth injection agglutinated three strains of Flexner bacilli in a dilution of 1:250 and would not agglutinate Bact. coli at that dilution. Precipitin tests with this rabbit's serum and a bacteriolytic filtrate were positive in a dilution of 1:2, and negative at 1:20.

SUMMARY

The filtrates of the stools of infants suffering from bacillary dysentery (Flexner), acute intestinal indigestion, otitis media and the need for regulation of feeding were bacteriolytic for one or more of twenty-seven strains of Flexner Shiga and typhoid bacilli. The technique by which these filtrates were obtained and tested, is described. D'Herelle's phenomenon is apparently non-specific and a bacteriolysant from a patient's stool may not be as active against the organism causing that patient's disease as against other strains. It does not necessarily play a part in the immunity or defense-mechanism of the body for

an active bacteriolysant was obtained from the stool of a patient the day before his death. One cannot at present predict which organisms will be attacked by any given filtrate, nor explain why certain cultures are readily lysed while others are unaffected. The bacteriolytic activity of a bacteriolysant may or may not be increased by passage through live dysentery cultures but it is decreased by passage through sterile peptone water and dead dysentery bacilli. Contamination and refiltration may increase or decrease the potency of a filtrate. Heating to 60 to 67°C. for forty-five to sixty minutes has little or no effect upon the activity of a filtrate. Bacteriolysants produce lysis more completely in low dilutions and at a pH of 8.0 and 8.2. Saline and peptone water suspensions of Flexner bacilli at pH 8 were equally lysable. Young cultures are lysed more readily than older ones. The addition of 1 cc. of N sodium hydroxide to 4 cc. of a bacteriolysant destroyed its bacteriolytic activity. Bacteriolysants were non-pathogenic for a rabbit and as I have previously reported (Davison, 1922b) had no therapeutic effect when administered to twelve young children suffering from bacillary dysentery.

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OBSERVATIONS ON THE NATURE OF BACTERIOLY-  
SANTS (D'HERELLE'S PHENOMENON, BACTERIO-  
PHAGE, BACTERIOLYTIC AGENT, ETC.)

PART II<sup>1</sup>

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From a consideration of the properties of bacteriolytic filtrates of stool cultures, which I have discussed in part I as well as in a review of the whole subject (Davison, 1922) it would seem possible that the bacteriolytic principle might be an enzyme, although as d'Herelle (Davison, 1922) has pointed out, none of the evidence absolutely disproves his contention that a bacteriophage is a living ultra-microscopic filter-passing organism. If then, one assumes that the substance is enzymatic, and that it can only be propagated from generation to generation by passage through living cultures one must conclude that it is contained in the bacterial cells or is a product of their metabolism. I therefore studied the properties of the colonies of "sensitive" and "normal" strains of *Bact. dysenteriae* (Flexner).

STUDY OF THE COLONIES OF A "SENSITIVE" STRAIN OF *BACT.*  
*DYSENTERIAE* (FLEXNER)

One-tenth cubic centimeter of a bacteriolytant was dropped on the surface of a five-hour agar culture of normal Flexner Y bacilli (Hiss and Russell) and the plate was replaced in the incubator at 37°C., for twenty-four hours. At the end of this time, the area bathed by the bacteriolytant was apparently devoid of growth or else covered with a fine film, while the remainder of the plate

<sup>1</sup> Presented at the twenty-third annual meeting, Society of American Bacteriologists, December 29, 1921. From the Department of Pediatrics of the Johns Hopkins University and the Harriet Lane Home of the Johns Hopkins Hospital, Baltimore, Maryland.

was covered with typical round dysentery colonies. This "bare" area was subcultured on another agar plate. After twenty-four hours at 37°C. this plate contained in equal proportions, small, regular, round, typical dysentery colonies and large mucoid colonies with very irregular outlines and a "moth eaten" appearance. Some were crescentic in shape, others triangular, and still others somewhat round with deep indentations in the edges. The edges of many of these colonies were more dense and refractile than their centers and appeared white and opaque in contrast to the gray translucency of the center. They resembled in every way, the photographs of "sensitive" colonies that Wollstein (Davison, 1922) has published. Forty-two successive subcultures of these "moth eaten" colonies, each gave rise to 10 to 100 per cent of the "moth eaten" colonies and 0 to 90 per cent of the small regular round colonies. Press of work prevented carrying these subcultures to more than forty-two generations.

Occasionally subcultures of the "moth eaten" colonies failed to grow in peptone water or on agar and reinoculation with several "moth eaten" colonies was necessary. When a "moth eaten" colony was subcultured in peptone water for one or two transfers, and then plated on agar, the plates frequently remained sterile or else contained nothing but regular colonies indicating the influence that a change in media might play in the multiplication of one or the other of these types. Gram-stained smears of these "moth eaten" colonies were largely composed of short fat Gram-negative bacilli with very few long forms. They were decolorized with more difficulty than smears of normal colonies. The "moth eaten" colonies represent the "sensitive" or "lysogenic" strain and the small, regular, round colonies the "resistant" strain described by others (Davison, 1922).

FILTRATES OF PEPTONE WATER CULTURES OF A "SENSITIVE"  
STRAIN OF BACT. DYSENTERIAE (FLEXNER)

The growth from twelve "moth eaten" colonies of the eighth generation of the "sensitive" strain of Flexner bacilli described above was inoculated into 500 cc. of peptone water (table 10).



This culture was then incubated at 37°C. for eighteen hours and 150 cc. of it was filtered. This filtrate was strongly bacteriolytic for normal Flexner bacilli. The remaining 350 cc. was then centrifuged at high speed and the supernatant fluid filtered. This filtrate was also strongly bacteriolytic. The sediment of

TABLE 10

*Bacteriolytic activity of filtrates of peptone water cultures of a "sensitive" strain of Bact. dysenteriae (Flexner Y, Hiss and Russell)*

FILTRATE OR SUSPENSION USED	BACTERIOLYTIC ACTIVITY OF FILTRATE AGAINST CULTURES OF NORMAL FLEXNER BACILLI	PRESENCE OF "SENSITIVE" COLONIES IN AGAR SUBCULTURES OF DYSENTERY BACILLI AFTER THEY HAD BEEN INCUBATED WITH THE FILTRATE FOR TWENTY-FOUR HOURS AT 37°C.
Filtrate of eighteen-hour peptone water culture of the eighth generation of a "sensitive" strain of Flexner bacilli	+	++
Filtrate of supernatant fluid of an eighteen-hour peptone water culture of the eighth generation of a "sensitive" strain of Flexner bacilli after the organisms had been centrifuged out	+	++
Saline suspension of the organisms of an eighteen-hour culture of the eighth generation of a "sensitive" strain of Flexner bacilli, after they had been centrifuged out and washed twice with saline. This suspension had stood at room temperature for 35 days to allow the organisms to disintegrate	±	++
Filtrate of ninety-six-hour peptone water culture of the thirty-fourth generation of a "sensitive" strain of Flexner bacilli	+	++
Sterile 1 per cent peptone water (control)	0	0

organisms was washed twice with 15 cc. of 0.9 per cent saline and then suspended in 5 cc. of saline. This suspension was left at room temperature for thirty-five days. Subcultures were then sterile. This suspension was also bacteriolytic. In other words, the lytic principle is both extracellular and intracellular for the fluid medium in which a "sensitive" strain was grown and also

the saline in which the washed organisms had been suspended and then allowed to disintegrate, were both bacteriolytic.

The growth from several "moth eaten" colonies of the thirty-fourth generation of the "sensitive" strain described above was inoculated into 100 cc. of peptone water. This culture was incubated ninety-six hours at 37°C. and then filtered. This filtrate was as strongly bacteriolytic as that of the eighth generation. This bears out Bordet's and Ciueca's (Davison, 1922) statement that "sensitive" strains are lysogenic for numerous generations.

FILTRATES OF AGAR CULTURES OF A "SENSITIVE" AND A NORMAL STRAIN OF BACT. DYSENTERIAE (FLEXNER)

The twenty-four hours' growth of "moth eaten" colonies on five agar plates of the fifth generation of the "sensitive" strain of Flexner bacilli described above was suspended in 20 cc. of N/10 phosphate solution at pH 8<sup>2</sup> and centrifuged. The supernatant phosphate solution was then filtered. This filtrate was strongly bacteriolytic (table 11). The sediment of organisms, which was a mass of mucoid, stringy material, was resuspended in 20 cc. of N/10 phosphate solution and ground up in a rotary agate mortar.<sup>3</sup> This suspension was then filtered. This filtrate was also strongly bacteriolytic (table 11). In other words the phosphate solution in which the sensitive organisms were washed and also the solution which contained the products of the ground up organisms were both bacteriolytic, suggesting that the lytic principle was extracellular as well as intracellular.

The twenty-four hours' growth of regular colonies on nine agar plates of a normal strain of Flexner bacilli was suspended in 20 cc. of N/10 phosphate solution at pH 8 and ground up in a rotary agate mortar. This suspension was then filtered. This filtrate had no bacteriolytic activity (table 11).

<sup>2</sup> N/10 Na<sub>2</sub>HPO<sub>4</sub>, 1.95 parts, and N/10 KH<sub>2</sub>PO<sub>4</sub>, 0.05 part. This solution itself was not bacteriolytic.

<sup>3</sup> The grinding was done by Dr. L. B. Lange of the Johns Hopkins School of Hygiene, to whom my thanks are due.

An attempt was made to break up saline suspensions of the twenty-four hours' growth on agar of Flexner bacilli and also of *B. subtilis* by alternate freezing and thawing but though the suspensions were frozen and thawed once or twice a day for thirty-five days, smears and cultures indicated that no damage

TABLE 11

*Comparison of the bacteriolytic activity of filtrates of agar cultures of a "sensitive" and a normal strain of Bact. dysenteriae (Flexner Y, Hiss and Russell) suspended in N/10 phosphate solution of pH 8 and ground in a rotary agate mortar*

FILTRATE USED	BACTERIOLYTIC ACTIVITY OF FILTRATE AGAINST CULTURES OF NORMAL FLEXNER BACILLI	PRESENCE OF "SENSITIVE" COLONIES IN AGAR SUBCULTURES OF DYSENTERY BACILLI AFTER THEY HAD BEEN INCUBATED WITH THE FILTRATE FOR SEVENTY-TWO HOURS AT 37°C.
Filtrate of the N/10 phosphate solution in which the growth from a twenty-four-hour agar culture of the fifth generation of a "sensitive" strain had been washed, i.e., in which the organisms had been suspended and then centrifuged out	+	++
Filtrate of the N/10 phosphate solution suspension of the washed organisms of a twenty-four hour agar culture of the fifth generation of a "sensitive" strain after they had been ground up in a rotary agate mortar	+	++
Filtrate of the N/10 phosphate solution suspension of the unwashed organisms of a twenty-four-hour agar culture of a <i>normal</i> strain of Flexner bacilli after they had been ground up in a rotary agate mortar	0	0
N/10 phosphate solution of pH 8 (control)	0	0

had been done to the organism. This is in marked contrast to the death of "sensitive" bacilli suspended in saline, at room temperature for thirty-four days (*vide supra*). Attempts were also made to dissolve dysentery bacilli by the addition of sodium hydroxide and of trypsin but the amount of the former required was sufficient to destroy any lytic activity that might have been present (table 9 part I,) and trypsin would not dissolve the organisms (table 15).

TABLE 13  
*The occurrence of "irregular" colonies in platings of normal strains of Bact. dysenteriae (Flexner)*

STRAIN OF BACT. DYSENTERIAE (FLEXNER) SUBCULTURED	AGE AND MEDIA OF CULTURE WHICH WAS SUBCULTURED	NUMBER OF "IRREGULAR" COLONIES IN SUBCULTURE PLATE AFTER TWENTY-FOUR HOURS AT 37° C.	NUMBER OF GENERATIONS TO WHICH "IRREGULAR" COLONY WAS CARRIED
Flexner Y (Hiss and Russell) (obtained from the Lister Institute, London)	3-hour peptone water culture	Several	Subculture contained 1 "irregular" colony; not carried farther
Flexner Y (Hiss and Russell) (obtained from the Lister Institute, London)	4-day peptone water culture	0	
Flexner Y (Hiss and Russell) (obtained from the Lister Institute, London)	136-hour peptone water culture	1	Subculture contained no "irregular" colonies
Flexner Y (Hiss and Russell) (obtained from the Lister Institute, London)	145-hour peptone water culture	0	
Flexner Y (Hiss and Russell) (obtained from the Lister Institute, London)	153-hour peptone water culture	1	Subculture contained 2 "irregular" colony; not carried farther
Flexner Y (Hiss and Russell) (obtained from the Lister Institute, London)	6-day peptone water culture (containing 1 per cent lactose)	Several	Subculture contained no "irregular" colonies
Flexner Y (Hiss and Russell) (obtained from the Lister Institute, London)	43-day peptone water culture (containing 1 per cent lactose)	Several	Subculture contained several "irregular" colonies; not carried farther
Flexner Y (Hiss and Russell) (obtained from the Lister Institute, London)	27-day agar culture	Several	Two successive subcultures each contained 2 "irregular" colonies; not carried further

Flexner Y (Ledingham) (obtained from Dr. E. G. D. Murray, London)	55-day peptone water culture	2	Subculture contained no "irregular" colonies
Flexner Y (Ledingham) (obtained from Dr. E. G. D. Murray, London)	58-day peptone water culture	0	
Flexner Y (Ledingham) (obtained from Dr. E. G. D. Murray, London)	6-day peptone water culture (containing 1 per cent lactose)	Several	Nine successive subcultures each contained several "irregular" colonies
Flexner Y (Ledingham) (obtained from Dr. E. G. D. Murray, London)	42-day peptone water culture (containing 1 per cent lactose)	Several	Two successive subcultures each contained several "irregular" colonies; not carried farther
Flexner Y (Ledingham) (obtained from Dr. E. G. D. Murray, London)	26-day agar culture	1	Two successive subcultures each contained several "irregular" colonies; not carried farther
Flexner Y (Ledingham) (obtained from Dr. E. G. D. Murray, London)	20 (?) -day agar culture	Several	Not subcultured
Flexner Y (Sada Gopall) (obtained from Dr. E. G. D. Murray, London)	55-day peptone water culture	Several	Two successive subcultures each contained several "irregular" colonies
Flexner Y (Sada Gopall) (obtained from Dr. E. G. D. Murray, London)	62-day peptone water culture	Several	Three successive subcultures each contained several "irregular" colonies
Flexner Y (Sada Gopall) (obtained from Dr. E. G. D. Murray, London)	6-day peptone water culture (containing 1 per cent lactose)	Several	Ten successive subcultures each contained several "irregular" colonies; not carried farther
Flexner Y (Sada Gopall) (obtained from Dr. E. G. D. Murray, London)	26-day agar culture	Several	Three successive subcultures each contained several "irregular" colonies; not carried farther
Flexner Y (Sada Gopall) (obtained from Dr. E. G. D. Murray, London)	20 (?) -day agar culture	Several	Not subcultured

TABLE 12—Continued

STRAIN OF BACT. DYSENTERIAE (FLEXNER) SUBCULTURED	AGE AND MEDIA OF CULTURE WHICH WAS SUBCULTURED	NUMBER OF "IRREGULAR" SUBCULTURE PLATE AFTER TWENTY-FOUR HOURS AT 37°C.	NUMBER OF GENERATIONS TO WHICH "IRREGULAR" COLONY WAS CARRIED
Flexner 115. (isolated from patient in H. L. H.)	20 (?) -day agar culture	Several	Not subcultured
Flexner 119. (isolated from patient in H. L. H.)	20 (?) -day agar culture	Several	Not subcultured
Flexner 120. (isolated from patient in H. L. H.)	20 (?) -day agar culture	Several	Subculture contained several "irregular" colonies
Flexner 90. (isolated from patient in H. L. H.)	1-day peptone water culture	0	
Flexner 90. 123, 137, 139, 140 and 141. (Isolated from patients in H.L.H.)	3-day peptone water culture	0	
Flexner W (stock culture); Flexner 120, and 131. (isolated from patients in H. L. H.)	4-day peptone water culture	0	
Flexner 90, 107, 123, 129, 131 and 141. (isolated from patients in H.L.H.)	20 (?) -day agar culture	0	

## STUDY OF THE COLONIES OF NORMAL STRAINS OF BACT. DYSENTERIAE (FLEXNER)

In sixteen out of twenty agar subcultures of four old laboratory strains of normal Flexner bacilli as well as in three out of eighteen agar subcultures of twelve freshly isolated strains of normal Flexner bacilli, I have noted one or more colonies with irregular

TABLE 13

*Bacteriolytic activity of filtrates of peptone water, cultures of normal dysentery bacilli (Flexner)*

STRAIN OF FLEXNER DYSENTERY BACILLUS	TYPE OF COLONY	NUMBER OF CULTURES FILTERED	LENGTH OF INCUBATION AT 37°C BEFORE FILTRATION	NUMBER OF ACTIVE FILTRATES	PERCENTAGE OF ACTIVE FILTRATES
Flexner Y (Hiss and Russell) (obtained from the Lister Institute, London)	Regular	8	5 hours to 152 days	4	50
Flexner Y (Ledingham) (obtained from Dr. E. G. D. Murray, London)	Regular	5	1 to 91 days	2	40
	Irregular*	2	1 day	2	100
Flexner Y (Sada Gopall) (obtained from Dr. E. G. D. Murray, London)	Regular	5	1 to 91 days	4	80
	Irregular	3	1 to 15 days	3	100
Flexner 106 (isolated from patient in H.L.H.)	Regular	1	5 days	1	100
Flexner 120 (isolated from patient in H. L. H.)	Regular	1	1 day	0	0
	Irregular	1	1 day	1	100
Total	Regular	20	5 hours to 152 days	11	55
	Irregular	6	1 to 15 days	6	100

\* Colonies with irregular edges that are sometimes present in subcultures of normal strains.

edges among the mass of regular, round, typical, dysentery colonies (table 12). These colonies were not as "irregular" and "moth eaten" as those which were noted in subcultures of "sensitive" strains but nevertheless resembled them. Successive

subcultures of some of these spontaneously "irregular" colonies contained "irregular" colonies for as long as ten generations. Subcultures of others contained nothing but regular colonies. The "irregular" colonies did not appear to be as viable as normal colonies and subcultures occasionally failed to grow. Inasmuch as these "irregular" colonies occurred more frequently in subcultures of old laboratory strains than of freshly isolated strains, it is possible that aging may influence their development. Growth in media containing lactose also appeared to favor their development. Filtrates of cultures of these "irregular" colonies were somewhat more lytic than filtrates of normal cultures (table 13). It is possible that these "irregular" colonies of normal strains and the "moth eaten" colonies of "sensitive" strains are related or even perhaps identical.

The different degrees of lysis produced in different cultures by the same filtrate (part I) apparently did not depend altogether on the presence or absence of "irregular" colonies in these cultures as only in six strains were "irregular" colonies noted.

FILTRATES OF PEPTONE WATER CULTURES OF NORMAL STRAINS OF  
BACT. DYSENTERIAE (FLEXNER)

Inasmuch as the bacteriolytic activity of stool filtrates, which had become contaminated with stool or air organisms and had then been refiltered, was sometimes increased, it seemed possible that filtrates of normal cultures themselves might be bacteriolytic. I therefore inoculated regular and "irregular" colonies of several strains of normal Flexner bacilli into peptone water and filtered these cultures after various periods of incubation at 37°C. (table 13). Eleven of the twenty filtrates (55 per cent) of peptone water cultures of regular colonies of five normal strains of Flexner bacilli and all of the six filtrates (100 per cent) of "irregular" colonies were slightly bacteriolytic. The degree of bacteriolysis was much less than that of stool filtrates and of filtrates of cultures of "sensitive" strains. In none of the instances in which subcultures were made of dysentery bacilli which had been incubated with these filtrates of normal cultures were "sensitive" colonies found. It is therefore probable that although filtrates



of normal strains of dysentery bacilli were bacteriolytic their activity was extremely weak.

BACTERIOLYTIC ACTIVITY OF FILTRATES OF A PEPTONE WATER CULTURE OF BACT. SUBTILIS

Nicolle (Davison, 1922) reported that cultures of *B. subtilis* and their filtrates, were bacteriolytic for several types of organisms. As he had not tested the bacteriolytic activity of *B. subtilis* against Flexner bacilli, I did so. As shown in table 14, the filtrate of a culture of *B. subtilis* (obtained from a contaminated plate) was not bacteriolytic for Flexner bacilli but the

TABLE 14  
*Bacteriolytic activity of filtrates of peptone water cultures of B. subtilis*

FILTRATE OR SUSPENSION USED	BACTERIOLYTIC ACTIVITY OF FILTRATE OR SUSPENSION AGAINST NORMAL FLEXNER BACILLI
Filtrate of four-day peptone water culture of <i>B. subtilis</i>	0
Filtrate of peptone water culture of normal dysentery bacilli which had been incubated forty-eight hours with the filtrate of <i>B. subtilis</i> culture (above)	±
Filtrate of eighteen-day peptone water culture or normal dysentery bacilli which had been intentionally contaminated with <i>B. subtilis</i>	±

filtrate of a normal dysentery culture to which the filtrate of a *B. subtilis* culture had been added, or which had been intentionally contaminated with *B. subtilis*, was slightly bacteriolytic. However this bacteriolytic activity was no more marked than that of filtrates of normal dysentery bacilli to which nothing had been added (table 13) so it is improbable that *B. subtilis* or its filtrates can lyse Flexner bacilli.

ABSENCE OF BACTERIOLYTIC ACTIVITY IN TRYPSIN

As the optimum reaction for bacteriolysis apparently coincides with that of the enzyme, trypsin, I tested the bacteriolytic activity of solutions of trypsin and also compared the action on gelatin of trypsin, bacteriolytants and "sensitive" and normal strains of Flexner bacilli. 1 per cent and 5 per cent solutions of

trypsin (Fairchild and Foster) were made in normal saline and 20 per cent alcohol. These were proven sterile by culture. As

TABLE 15  
*Absence of bacteriolytic activity in trypsin*

SOLUTION OR FILTRATE USED	BACTERIOLYTIC ACTIVITY OF SOLUTION OR FILTRATE AGAINST CULTURES OF NORMAL DYSENTERY BACILLI	PRESENCE OF "SENSITIVE" COLONIES IN AGAR SUBCULTURES OF DYSENTERY BACILLI AFTER THEY HAD BEEN INCUBATED WITH SOLUTION OR FILTRATE FOR TWENTY-FOUR HOURS AT 37°C.
1 per cent alcoholic solution of trypsin	0	0
1 per cent saline solution of trypsin	0	0
5 per cent alcoholic solution of trypsin	0	0
5 per cent saline solution of trypsin	0	0
F 104 = 5 cc. 5 per cent alcoholic solution of Trypsin added to 100 cc. of an eighteen-hour peptone water culture of Flexner Y bacilli; incubated eighteen hours and then filtered	0	0
F 103 = 2 cc. 5 per cent saline solution of Trypsin, added to 100 cc. of an eighteen-hour peptone water culture of Flexner Y bacilli; incubated eighteen hours and then filtered	0	0

TABLE 16  
*Absence of gelatin liquefying enzyme in bacteriolysants and cultures of "sensitive" and "normal" strains of Bact. dysenteriae (Flexner Y) (Hiss and Russell)*

BACTERIOLYSANT, CULTURE OR SOLUTION USED	LIQUIFICATION OF GELATIN AT ROOM TEMPERATURE
F 100 (actively bacteriolytic filtrate)	0 (34 days)
"Moth eaten" colonies of "sensitive" strain of Flexner Y bacilli	0 (48 days)
Regular colonies of normal strain of Flexner Y bacilli	0 (48 days)
5 per cent alcoholic solution of trypsin	+ (24 hours)
5 per cent saline solution of trypsin	+ (24 hours)

shown in table 15, neither these solutions, nor filtrates of dysentery cultures which had been incubated with these solutions were bacteriolytic. That the trypsin used was active and could liquefy gelatin is shown in table 16.

ABSENCE OF GELATIN LIQUEFYING ENZYME IN BACTERIOLYSANTS  
AND CULTURES OF "SENSITIVE" AND "NORMAL" STRAINS OF  
BACT. DYSENTERIAE (FLEXNER Y, HISS AND RUSSELL)

As shown in table 16, gelatin was not liquefied by a bacteriolysant nor by cultures or "moth eaten" colonies of a "sensitive" strain of Flexner bacilli nor by regular colonies of a normal strain.

SUMMARY

"Moth eaten" or "sensitive" colonies were obtained in subcultures of dysentery bacilli which had been attacked by a bacteriolysant. They were subcultured for forty-two successive generations. The media in which "sensitive" strains of Flexner bacilli were grown, the solutions in which they were washed, and the suspensions of disintegrated or ground up "sensitive" bacilli, were all strongly bacteriolytic. In agar subcultures of old laboratory and freshly isolated normal strains of Flexner bacilli "irregular" colonies were occasionally found, which resembled the "moth eaten" colonies of "sensitive" strains. Filtrates of peptone water cultures of normal strains and especially of their "irregular" colonies, were slightly bacteriolytic. *B. subtilis* was not bacteriolytic for Flexner bacilli. Trypsin was not bacteriolytic and bacteriolysants and "sensitive" and "normal" strains did not liquefy gelatin.

CONCLUSION

It would seem possible that the lytic principle in d'Herelle's phenomenon is an enzyme. This enzyme is not trypsin. A small amount of the lytic principle is contained in, or produced by, normal strains of Flexner bacilli which have been out of contact with the human body for many years. It is possible that the amount of the bacteriolytic enzyme produced by a culture can be increased by aging, by growth in special media or by contact with external influences such as intestinal secretions, tissue extracts, leucocytes, etc. The action of these external influences is probably to favor the development of lysogenic organisms at the

expense of the non-lysogenic. This enzyme not only dissolves organisms but also favors the multiplication of bacteria which produce this enzyme. In this way the bacteriolytic principle is carried from generation to generation. It is highly improbable that this phenomenon represents a defense mechanism on the part of an animal against bacterial invasion.

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# CLOSTRIDIUM PUTRIFICUM (B. PUTRIFICUS BIENSTOCK), A DISTINCT SPECIES

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There has been much confusion regarding the identity of the organism which was discovered by Bienstock (1884) and which he named *Bacillus putrificus*. According to his own admission, Bienstock for a while worked with impure cultures of this anaerobe. However, he described its morphology and putrefactive properties, in his first publication (1884), and at a later date published a more complete description (1899).

Bienstock described his *B. putrificus* as a long, slender rod with a terminal spore. His photographs show both round and more or less oval spores. Some investigators have interpreted his phrase "baguette de tambour" and the word "Trommelschlägenform" as meaning literally *drumstick* and implying an oval form of the spore. This interpretation does not appear to the writers to be correct. In its sporulating stage *Clostridium tetani* is described quite generally in the literature as a drumstick form, and this term is generally applied to organisms possessing a round, terminal spore. Tissier and Martelly (1902) liken the spore of *C. putrificum* to that of *C. tetani*. Rettger (1906) also points out a strong resemblance, and further draws attention to the ease with which *C. putrificum* may, by morphology alone, be mistaken for the tetanus bacillus. However, certain writers have in recent years referred to *C. putrificum* as producing a terminal oval spore. Even so late a work as that of the British Medical Research Committee (1917) has included this organism among the putrefactive anaerobes which develop such spores. Some American writers have fallen into the same error. Sturges and Rettger (1919), on the other hand, state emphatically as the result of a continued study of several pure

strains which had been isolated by recently devised methods, that the spores of this organism are round.

The writers have employed the strains isolated by Sturges, in their morphological, biochemical and cultural studies of *C. putrificum*, and have corroborated the earlier observations of Rettger and those of Sturges and Rettger, particularly the points regarding morphology. In all of the stock strains ex-



FIG. 1. SHOWING RODS, CHAINS AND CHARACTERISTIC DRUM STICKS OF *C. putrificum*. INCUBATED 3 WEEKS AT 37°C. IN EGG-MEAT MEDIUM. FUCHSIN STAIN.  $\times 1000$

amined (four) the microscopic appearances of the organism were the same. The bacilli were long and slender, frequently slightly curved, and in the sporulated condition possessed a terminal, round spore which in the fully developed drumsticks appeared very large as compared with the thickness of the rods. The rods were as a rule longer and much more slender than those of *C. tetani* grown on the same medium (fig. 1). Colonies also are characteristic (fig. 2).

There has been considerable discussion as to whether *C. putrificum* attacks carbohydrates. Bienstock did not deal with this phase in his earlier work. Tissier and Martelly (1902) showed that it does not act upon glucose, or at most only slightly. In a later publication (1906) Bienstock confirms the observations of Tissier and Martelly, and of Rodella (1905), and holds that *C. putrificum* does not attack any of the carbohydrates, and that the minute quantities of acid formed in sugar media arise from the decomposition of protein. This organism occupies a unique position, therefore, among the anaerobes, in that it attacks proteins but not carbohydrates appreciably if at all. Bienstock's

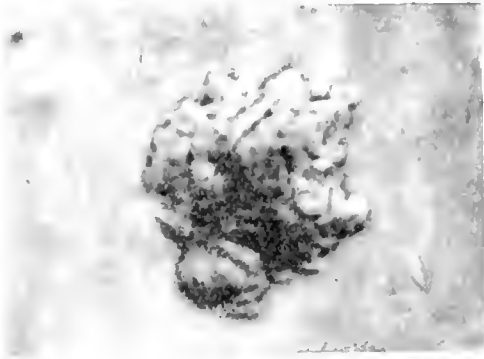


FIG. 2. COLONY ON 1.6 PER CENT GLUCOSE AGAR. INCUBATED 10 DAYS AT 37°C.

"*B. paraputrificus*," on the other hand, did attack carbohydrates with acid and gas formation.

In the recent reports of the British Medical Research Committee, *C. putrificum* is described as being saccharolytic to the extent of acting upon glucose, maltose, lactose, sucrose and starch. They even go further (1919) and consider it to be, not an entity or distinct species, but a mixture perhaps of *C. sporogenes* and *C. tertium* or of *C. sporogenes* and *C. cochlearius*.

In our own study of this organism its unique position appears to be so clearly defined and its characters are found to be so outstanding that we see no reason for mistaking it for any other anaerobe or mixture of anaerobes. Because of the fact that

the above mentioned work of the British Medical Research Committee has exerted and is exerting a commanding influence on present-day attempts at re-classification of the anaerobes, particularly those which were found in war wounds, we feel prompted to defend *C. putrificum* (*B. putrificus*) as a distinct species and to attempt to place it in its proper classification group.

*C. putrificum* bears a more or less close resemblance to *C. tetani*, and *C. tetanoides* morphologically, but differs markedly

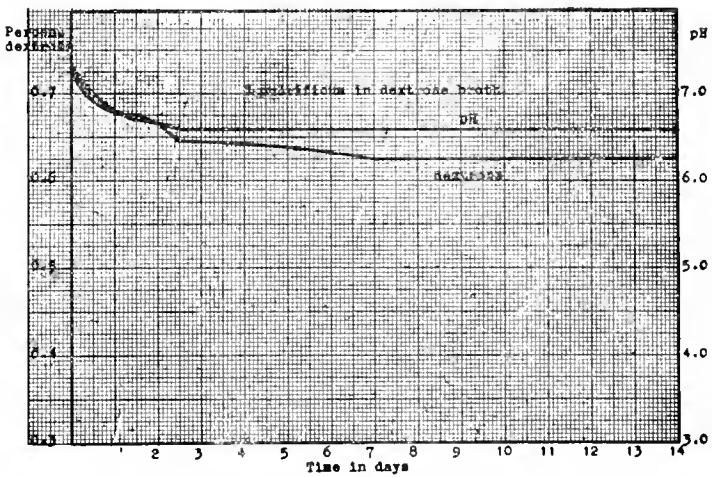


CHART I. PLOTTINGS OF pH AND GLUCOSE FIGURES FOR *C. putrificum* IN GLUCOSE BROTH

from them in certain important respects. Pure strains of *C. tetani* and *C. tetanoides* are non-putrefactive, and are unable to digest meat or egg-meat medium even during months of incubation. *C. tetani* is pathogenic, whereas the other two organisms are entirely void of this property. Furthermore, *C. putrificum* does not attack carbohydrates or at the most exerts but a feeble action. No gas was produced from any of the 25 carbohydrates, alcohols and glucosides employed by us, and only slight amounts of acid from three or four, one of these being glucose. Quantitative estimations of the amounts of glucose consumed were made, and the results expressed in plotted curves (chart I).



This organism constitutes a unique division, therefore, among the known anaerobes, and differs from all others in that it is powerfully putrefactive, but non-saccharolytic, or at the most but feebly saccharolytic.

While *C. putrificum* is an active proteolytic and putrefactive organism, it differs from *C. sporogenes* in that in pure culture in meat or egg-meat medium it develops slowly and produces little or no apparent change in less than a week or ten days, even at the most favorable temperature. After the long incubation period, however, the protein is vigorously attacked with the formation of the usual putrefaction products. When mixed with other organisms, as for example *Staphylococcus aureus* or *Proteus vulgaris*, the preliminary period is relatively short and putrefactive decomposition takes place much sooner. These observations are in accord with those of Sturges and Rettger (1919).

*C. putrificum* has a very marked peptolytic action in ordinary peptone broth, as may be shown readily with the aid of the ammonia, Sørensen, Van Slyke and quantitative biuret tests. The complex nitrogenous substances in the commercial peptone are destroyed rapidly, with the formation of large amounts of ammonia, but with little permanent increase in amino nitrogen. The biuret figures drop sharply (chart 2).

Important points of difference between *C. putrificum* and *C. sporogenes*, *C. tertium* and *C. cochlearius*, organisms with which the Bienstock anaerobe has been confused, are as follows: *C. sporogenes* is actively proteolytic and saccharolytic; *C. tertium* is saccharolytic and peptolytic, but not proteolytic; and *C. cochlearius* (according to the statements of the British Medical Research Committee) is saccharolytic and non-proteolytic. *C. putrificum* occupies an entirely different position in that it is proteolytic and but very slightly or not at all saccharolytic. There are sufficient morphological and cultural differences to set the Bienstock anaerobe apart from the others, and not only should it be easy to distinguish *C. putrificum* from these three organisms, but from all known anaerobes by a combination of the morphological, cultural and biochemical characters, which have been mentioned above.

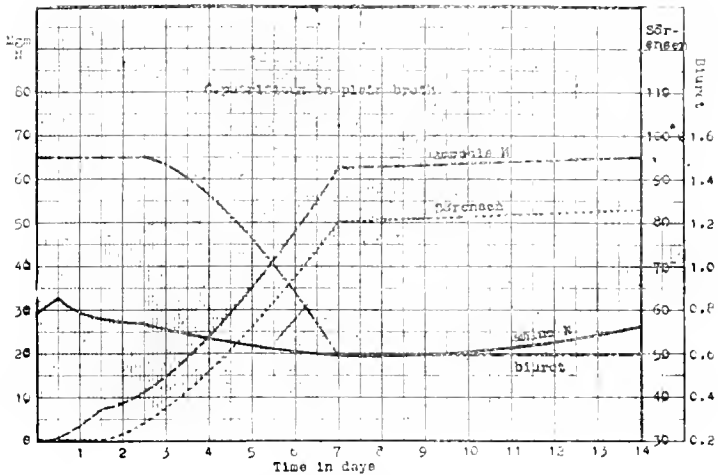


CHART 2. PLOTTINGS OF AMMONIA, SØRENSEN, AMINO NITROGEN AND BIURET FIGURES FOR *C. putrificum* IN PLAIN BROTH

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## TRANSPARENT MILK AS A BACTERIOLOGICAL MEDIUM

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The addition to milk of an oxalate or a citrate, in the form, for example of a sodium salt, will cause the opacity of milk to disappear and give a solution which is opalescent in thick layers but almost clear in thin layers.<sup>1</sup> A similar result may be obtained with sodium sulphate when added in relatively larger amounts. This change is particularly evident when the milk is diluted slightly. Although we have not found the phenomenon described in the literature it may have been observed by those who for infant feeding have added sodium citrate to milk to prevent rennet coagulation. The general nature of the reaction has been known and the procedure has been used to prevent the coagulation of blood. Arthus (1902) showed that sodium citrate in the proportion of 2 to 3 parts per 1000 would prevent the coagulation of milk by rennin. Bosworth and Van Slyke (1914) in a study of "why sodium citrate prevents curdling of milk by rennin" found that there was an increase in the amount of soluble calcium which would pass through a Chamberland filter with increasing quantities of sodium citrate until approximately 1 gram of the hydrated salt had been added to 100 cc. of milk. Rennin coagulation was prevented when 0.4 gram of the citrate had been added to 100 cc. of milk. The failure to coagulate in the presence of citrates was ascribed to the formation of calcium citrate or sodium-calcium citrate.

The loss of opacity is apparently due to the removal of the calcium combined with casein or globulins in milk. The addition

<sup>1</sup> Howe, Paul E., unpublished work.

of oxalate or sulphate results in the formation of insoluble calcium oxalate or sulphate which may be removed by centrifugation. By the addition of sodium citrate, however, the calcium of milk is not precipitated (Arthus, 1902), but remains in solution in a non-effective form in so far as its ability to combine with casein or to react with the para-casein formed as a result of the action of rennin are concerned. We have not found an adequate explanation of the nature of the calcium-citrate combination. That it is in solution is indicated by the work of Arthus and of Bosworth and Van Slyke. We have evidence of this in the fact that during each sterilization there is formed a heavy precipitate of a calcium salt which we assume to be calcium citrate. It has the property of calcium citrate of being precipitated when heated and redissolving upon cooling. On the other hand, the calcium in solution in the clarified milk must be sufficient to form the insoluble para-casein compound for there is enough calcium in milk to do so and in the preparation of transparent citrated milk none need be removed. Sabbatani (1902) states that the effect of the citrate in blood is due to a reduction in the number of calcium *ions* in solution. He also shows that the ratio of citrate to calcium for the prevention of coagulation is three to one. Arthus holds that there is a specific effect of the citrate ion which inhibits flocculation, in addition to any changes which may take place in solubility.

In the preparation of transparent milk as a bacteriological medium we have diluted 1 part of skim milk with 2 parts of distilled water and then added 0.4 per cent of sodium citrate. After standing for about an hour the clarified milk may be filtered through paper though this is hardly necessary if the mixture is allowed to stand sufficiently long. To avoid caramelization of the milk sugar during sterilization the reaction of the medium is adjusted to about pH 6.8. The medium is then tubed and sterilized fractionally. During each steaming in the Arnold sterilizer a heavy precipitate is thrown down but redissolves as the medium cools. There finally results an almost water-clear medium without any precipitate whatever. Oxalated milk may be prepared in the same manner except that the fine precipitate of

calcium oxalate should be removed by centrifugation. For most purposes the citrated milk appears to be the more satisfactory medium, since the citrate gives changes which correspond with those which take place in milk without further treatment. Citrates are a normal constituent of milk.

We have used these media for the cultivation of a number of organisms—streptococci, anaerobes, and members of the colony-typhoid group. A few organisms grow better in the citrated milk than in oxalated milk, though the latter is useful for special purposes because of the removal from it of the calcium. The citrated milk shows all the cultural reactions which may be observed in untreated milk of the same dilution. Some organisms, notably the paratyphoids, produce a reaction not to be observed in untreated milk. These produce in citrated milk a milky translucence or opacity which we attribute to the decomposition of the citrate with liberation of calcium in the ionized state, an appearance which may be produced artificially by the addition of a small amount of calcium chloride to the sterile medium. This reaction is not produced by cultures in oxalated milk from which the calcium has been removed.

The cultural reactions which we have observed in transparent citrated milk may be summarized as follows:

- I. Neither acid production nor digestion of casein. Reaction alkaline or neutral
  - a. The medium remains clear except for the clouding due to visible growth. The addition of a few drops of  $\text{CaCl}_2$  solution causes it to become milky. Example: *Bact. typhosum*, *Bact. alkaligenes*
  - b. The medium becomes milky, probably due to a release of ionized calcium. Example: *Bact. paratyphosum*, *Bact. cholerae-suis*
- II. Acid production
  - a. A small amount of acid may do nothing more than change the color of the indicator which may be added to the medium. Example: *Strep. pyogenes*
  - b. A larger amount of acid may produce translucence. Often observed as a transitory reaction. Example: *Bact. cloacae*
  - c. Large amounts of acid produce coagulation or precipitation of casein. Example: *Strep. lacticus*, *Lactobacillus bulgaricus*, *Bact. coli*, *Clostridium welchii*
- III. Rennin production
  - a. Without release of calcium from citrate should give precipitate when calcium chloride is added unless the casein has been digested

b. With release of calcium from citrate should produce precipitate or coagulum

IV. Casein digestion

a. Should give diminished or negative precipitation upon addition of acetic acid. Example: *Proteus vulgaris*

Various combinations of the above reactions may be observed and there are doubtless other reactions which are not mentioned above, for instance, with alkali production we have sometimes observed a thickening of the medium into a transparent jelly. This reaction was produced by *Bact. alkaligenes*.

SUMMARY

Milk may be transformed into a transparent culture medium by the addition of small amounts of various salts. Sodium citrate seems to be the most suitable for this purpose.

In such a medium there may be observed not only the ordinary reactions of various bacteria in milk but also some others not observed in untreated milk.

The principal advantages of the transparent milk as a medium reside in the greater visibility of changes which occur in it. Indicators are much more easily seen in it than in opaque milk. As long as the acidity remains below pH 5.5 colorimetric hydrogen ion determinations are easily made. Clouding and the formation of sediment due to bacterial growth may be observed as in bouillon when no visible change whatever is produced in ordinary opaque milk.

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# THE USE OF AGAR SLANTS IN DETECTING AMMONIA PRODUCTION AND ITS RELATION TO THE REDUCTION OF NITRATES

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The use of agar slants in detecting the production of acid by bacteria has been reported by our laboratory (Conn and Hucker, 1920), and used successfully for some time. The determination of such physiological activities on solid media has many advantages over the older methods in which liquid cultures were used and especially is this true in the case of many of the free-living organisms which either fail to grow or grow very poorly in liquid media. As ammonia production by bacteria has an important significance in assigning organisms to their natural groups, an effort has been made to adapt some of the many ammonia tests to use with solid media. One of the chief difficulties in employing the usual methods has been the fact that the presence of certain organic compounds interferes with the reaction and, as all cultural media contain more or less organic material, satisfactory results could not be obtained. This is especially true where Nessler's reagent is used as this has a special affinity for organic nitrogenous material especially the aldehydes. Because of this fact, it has been impossible to use glucose as a source of carbon in a medium in which bacteria are grown to be tested for ammonia production.

Bacteria in their metabolism are usually considered as securing their energy from the oxidation of various carbon compounds, while the nitrogen required to produce the bacterial protoplasm is derived from various organic sources and in some cases from inorganic compounds. The utilization of protein and other organic sources of nitrogen, whether for energy or for protoplasm building, is in many cases accompanied by free ammonia pro-

duction. Consequently, the presence or absence of this particular by-product of bacterial metabolism indicates fundamental activities of the bacterial protoplasm.

In the determination of ammonia production the Thomas (1912) test, which has been used in part of Ayers, Rupp and Mudge (1921), in liquid cultures for studying certain strains of streptococci, has been found to be exceedingly helpful in our laboratory either in the presence or absence of organic matter.

The organisms to be tested are grown on the following medium:

Peptone .....	4.0 per cent
Glucose .....	0.2 per cent
Dipotassium phosphate .....	0.5 per cent
Agar .....	15.0 grams
Water .....	1000.0 cc.

The culture is incubated at the optimum temperature, preferably for one week, and 1 cc. of each of the following reagents added to the surface of the slant: 1 per cent phenol solution and sodium hypochlorite (1 per cent available chlorine). The tubes are allowed to stand for one-half hour and if ammonia is present, a decided blue color appears.

As an optional method, an adaptation of the common Sorensen method has also proved satisfactory for use with agar slants which contain no peptone or other organic nitrogen. As a reagent, 2 cc. of neutral formaldehyde (containing a few drops of phenolphthalein) are added to each slant. Ammonia production is indicated by the presence of acid which decolorizes the added reagent. The absence of ammonia is indicated by no change in the reaction of the fluid on the surface of the slant.

Neither of these tests are new; but their adaptation for use with solid media with free-living organisms, many of which fail to grow in liquid media, has been helpful in studying their physiological activities.

These tests are also of special importance in connection with the determination of nitrate reduction, for in some cases a negative nitrite reaction in a nitrate medium does not indicate failure to reduce nitrate. Some organisms may convert the nitrite as rapidly as it is formed either into ammonia or into the protein



of their own bodies. In the former case an ammonia determination may supply the missing information. The organisms to be thus tested may be grown either on a synthetic medium containing no nitrogen except nitrate, or if they fail to grow under such conditions on a nitrate-peptone medium. In the latter case a duplicate inoculation must be made on to a similar peptone medium without nitrate to be sure that the ammonia is not produced from the peptone. For a synthetic medium the writers recommend that described by the Committee on the Descriptive Chart (1920) except that glucose be substituted for sucrose as it is a more available sugar than the latter and its presence is not confusing when the Thomas test instead of the Nessler test is used to detect ammonia. This medium is:

Water.....	1.0 liter
Agar.....	15.0 grams
Glucose.....	10.0 grams
Potassium nitrate (other inorganic nitrogen sources may be used if desired).....	1.0 gram
Calcium chloride.....	0.5 gram
Dipotassium phosphate.....	0.5 gram

If these tests are used in routine work for those organisms that do not show nitrite as ordinarily tested in nitrate medium, much valuable information can be obtained. It has been found in this laboratory, for instance, that out of several hundred cultures examined about 60 were nitrite-negative when grown on nitrate-peptone agar. Of these 60 it was found, however, that 4 produced ammonia on the above synthetic media without any evidence of nitrite formation. By the usual methods such organisms would be classed as non-reducers, whereas in the absence of all other possible sources of ammonia, the positive ammonia-reaction plainly indicates that they must have reduced the nitrates to ammonia, and in such cases to class them as non-reducers would be a decided error.

As a routine procedure in connection with nitrate reduction such a combination of tests shows promise of giving interesting data on the physiological activities of bacteria.

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## METHODS OF PURE CULTURE STUDY

### REPORT OF COMMITTEE ON BACTERIOLOGICAL TECHNIC

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Several years ago the committee on the descriptive chart was asked by the Society to prepare a manual of methods to be used in connection with the chart, in the pure culture study of bacteria. As at that time the methods were felt to be very crude, and as considerable work was then in progress on them, it seemed that the best thing that could be done was merely to draw up a short report on the subject, which was printed in the *Journal* (Committee on Descriptive Chart, 1917) and was distributed in separate form with the charts that were ordered. A limited edition of these separates was printed with the expectation of getting out a revision shortly. Such a revision was prepared a few years later (1920) and at the present time there is need of a second revision.

This work has now been put in the hands of the more recently organized committee on bacteriological technic and this committee has drawn up at the present time a third edition of the report on methods of pure culture study which is to replace the second edition now almost out of print. So much material has been collected, however, in the last few years and the methods are now so much more satisfactory than they were when the first edition was prepared that it seems advisable to the committee to make this third edition a separately published manual of methods, according to the instructions given to the earlier committee.

This manual, as now prepared, contains to a large extent methods that have been printed in the earlier editions of the report; but for certain procedures, new methods have been described.

These new methods are now published to call them to the attention of the Society for discussion and criticism before the manual is printed. At the next meeting of the Society the committee expects to ask for approval in publishing this manual of methods.

When asking approval to print this manual, the committee is not to ask that the methods included therein be made official. The committee has consistently taken a stand against official methods for research work and does not wish that these methods be construed as such. For this reason the committee does not want the methods officially adopted by the Society, but merely wants approval for the plan of publishing them in a manual of methods recommended for pure culture study.

The following pages give the new methods that are included in this report.

#### SPECIAL STAINS

Of these the Gram stain has been given particular attention by the committee. In the last edition of the report on methods (1920) the Stirling modification was recommended; but in recent tests it has proved to be less satisfactory than certain other anilin oil methods. In the first place it has been found to have no special advantages as to keeping quality; and in the second place it gives preparations showing a large amount of precipitate; and in the third place a careful search through the literature has so far failed to show the original place of publication of this technic;<sup>1</sup> and finally, the methods employed by various bacteriologists under this name differ very greatly from one another. At present it seems best to recommend three different procedures. The first calls for Ehrlich's anilin gentian violet with the technic given in Buchanan's *Veterinary Bacteriology* (p. 103) and is the anilin oil formula which has given the best results in recent tests. The second method is the ammonium oxalate

<sup>1</sup> The committee will be very grateful to any one who can furnish information as to where this Sterling technic was originally published, if it was actually published, and if not, where it was originally described.

method (Hucker, 1922) and the third that of Atkins (1920), both of which are highly to be recommended on account of the keeping quality of the solutions, but have not yet been compared with the older methods sufficiently so that the committee can be sure that they can replace them.

*Method 1*

Ehrlich's anilin gentian violet<sup>2</sup>

Gentian violet (saturated alcoholic solution) . . . . .	6 cc.
Alcohol . . . . .	5 cc.
Anilin water (98 cc. water to 2 cc. anilin oil) . . . . .	50 cc.

Lugol's iodine solution

Iodine . . . . .	1 gram
Potassium iodide . . . . .	2 grams
Water . . . . .	300 cc.

*Method 2 (Hucker's)*

Gentian violet (saturated alcoholic solution) . . . . .	1 part
Ammonium oxalate (1 per cent aqueous solution) . . . . .	4 parts

Lugol's iodine solution (as usual)

*Method 3 (Atkins')*

Gentian violet (saturated alcoholic solution) . . . . .	1 part
Anilin sulphate (1 per cent aqueous solution) . . . . .	3 parts

Atkins' iodine solution

Iodine . . . . .	2 grams
Normal NaOH . . . . .	10 cc.
Water . . . . .	90 cc.

Technic. Stain one minute; treat in iodine solution one minute; decolorize one minute with methods 1 and 2, five minutes with method 3; counterstain about ten seconds. For the counterstain use 1:10 safranin (i.e., 1 part saturated alcohol solution to 10 parts water) 1:10 eosine or 1:10 bismark brown or pyronin. With method 1 do not wash between the different procedures; merely drain thoroughly with the other two methods wash between each step.

<sup>2</sup> The committee has found crystal violet to be a very satisfactory substitute for gentian violet in all these formulae. It is of much more constant composition, and therefore to be preferred to the very variable gentian violet. See the following paper, p. 533.

## FERMENTATION OF SUGARS, ALCOHOL AND GLUCOSIDES

This may be determined in either solid or liquid media according to which the organisms to be investigated prefer for their best growth; but the committee recommends that solid media be used whenever possible.

The method recommended for determining fermentation in solid media is that described by Conn and Hucker (1920).

When solid media are to be inoculated, use the following agar slant method: Prepare two lots of agar media, one the regular beef extract agar and the second a peptone-free agar having the following composition:

NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub> . . . . .	1.0 gram	Adjusted to pH 7 by the addition
KCl . . . . .	0.2 gram	of NaOH. About 6 cc. normal
Agar . . . . .	15.0 grams	NaOH required
Water . . . . .	1000	cc.

To both of these media add 1 per cent of the fermentable substance to be investigated and 2 cc. per liter of a saturated aqueous solution of brom cresol purple. Distribute these two media in test tubes and cool in a slanting position. The tubes should be inoculated either on the surface alone like an ordinary agar slant, or partly on the surface and partly in a stab at the base of the tube. The tubes should be inoculated at 37°C. or 25°C. according to the optimum temperature of the organism under investigation. Examine after twenty-four hours and then as often as seems necessary according to the rapidity of the growth so as not to overlook any changes in reaction. Acid can be readily determined by the yellowing of the indicator. A very good idea as to the amount of acid can be obtained by noticing the size of this zone of yellow. This method is only roughly quantitative yet gives fairly satisfactory results. A convenient method of denoting results is the following: use a single + sign if acid is produced but the yellow zone does not extend to the base of the slant, denote ++ if the acid zone extends to the base of the slant but no further, +++ if it extends half way from the base of the slant to the bottom of the tube and ++++ if the whole tube of agar has turned to its acid color. Use the symbol (0) to indicate no change in reaction.

Interpreting results the two media should be taken into account as some organisms produce more easily detectable acid on the peptone media, some on the peptone free media. In the former case it may be assumed that the acid produced by the organisms is obscured either by the buffer of the peptone or by the alkalinity produced from it.

If it is decided to observe the production of alkalinity as well as acidity, it is recommended that two indicators be used, e.g., brom cresol purple with cresol red (see 1920 report, p. 130). The use of these two indicators is decidedly recommended because in many cases it is instructive to observe the production of alkalinity. Some organisms produce acid in one part of the media and alkali in another; when this occurs it is to be noted. Denote alkali with a - sign.

In using solid media, the production of gas can be quite readily detected by the presence of bubbles and cracks in the agar. It would seem theoretically that this is a less accurate way of determining gas production than the fermentation tube; but wherever the two methods have been compared in the case of organisms growing well or better on solid media, agar slants have been found to give as reliable results as fermentation tubes.

When liquid media are to be inoculated, prepare two media similar in composition to the above mentioned but without agar, sterilize in fermentation tubes and incubate at 37° or 25° according to the optimum temperature of the organism in question. The media may contain the same indicator or indicators as those already mentioned in the case of solid media, in which case the cultures should be examined at intervals, as often as seems necessary to record changes in the reaction. Although there is ordinarily no appreciable influence of the indicator on the growth of the organism still there may be cases when the investigator prefers using media without an indicator; in this case the culture should be tested ordinarily on the first, third and seventh days, although the best days of testing will depend on the rapidity of growth of the organism. To test for acid, use an indicator having a range which covers the reaction of the culture. If possible compare the culture with a standard buffer solution.

## REDUCTION OF NITRATES

The following procedure is recommended: Inoculate first into nitrate broth and on to slants of nitrate agar (containing 0.1 per cent  $\text{KNO}_3$  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 in the broth or by cracks in the agar. Then test for nitrate 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  $\alpha$ -naphthylamine in 1 liter of 5 N acetic acid or of very dilute sulphuric acid (1 part concentrated acid to 125 parts water).

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 check which has been kept under the same condition to guard against errors due to absorption of nitrite from the air. Presence of nitrite or of gas shows the nitrate to have been reduced. 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; altering the reaction; adding some readily available carbohydrate. Presence of nitrite in any nitrate medium whatever should be recorded as nitrate-reduction. Presence of gas should be similarly interpreted, provided there is no other substance (e.g., sugar) present in the medium, from which the organism under investigation is able to produce gas.

In the case of those organisms which are nitrite-negative and produce no gas in nitrate-peptone agar or broth, but which show



good growth in one or both media, proceed as follows before concluding that the organisms do not reduce nitrate:

Inoculate into:

A. Nitrate-peptone agar or broth.

B. Peptone agar or broth without nitrate.

C. Synthetic nitrate medium (formula recommended: Nitrate 1 g,  $K_2HPO_4$  0.5 g,  $CaCl_2$  0.5 g, glucose 10 g, distilled water 1000 cc., with or without agar according to the organism under investigation).

D. Peptone agar with 2 p.p.m. potassium nitrite.

Prepare the following reagents for the Thomas test for ammonia:

1. A 5 per cent solution of phenol,

2. A solution of sodium hypochlorite containing one per cent available chlorine. To obtain this amount of available chlorine, the solution should be so adjusted that 1 cc. of it should neutralize 2.86 cc. of a N/10 solution of sodium thiosulphate (i.e., 24.8 grams to the liter), titrating with starch as an indicator in the presence of acetic acid and potassium iodide.

In making the Thomas test 1 cc. of each of the above reagents should be added to the broth or agar slant<sup>3</sup> on which the culture has been growing and allowed to stand half an hour. A blue color indicates the presence of ammonia.

After incubation, test A and B for ammonia by the Thomas method; test C for nitrite as usual, and for ammonia by the Thomas method; test D for nitrite. The results give the following indications:

On A: Presence of ammonia indicates nitrate reduction if no ammonia is present in B. Absence of ammonia on both media indicates non-reduction provided this conclusion is confirmed by the results on C and D. Otherwise inconclusive.

On C: Presence of either nitrite or ammonia indicates nitrate reduction. Absence of both, if the growth is good, strongly indicates non-reduction.

On D: Presence of nitrite indicates non-reduction in case this is confirmed by the two above tests, for if the organism cannot destroy

<sup>3</sup> This test can be made for this purpose to great advantage with agar slant culture as well as with liquid media, as recently shown by Hucker and Wall (1922).

the extremely small amount of nitrite present in D it is not likely to have destroyed the nitrite in nitrate agar as fast as it produced it.

The organisms for which inconclusive results must be recorded are those which produce ammonia in B, which fail to grow on C, and which destroy the nitrite in D. The number of organisms in this group is probably very small.

#### INDOL PRODUCTION

The indol test has always been one of the most unsatisfactory of those that have been used for characterizing bacteria. This has been true, partly because of the variable composition of the media used, and partly because of the inaccuracy of the tests used for detecting the presence of indol.

The first of these objections (i.e., the variable composition of the media) Zipfel (1912) has tried to avoid by using tryptophane in place of peptone. As this medium is not satisfactory for routine use other investigators have tried to accomplish the same purpose by using a solution of peptone treated with trypsin. Frieber (1921) for instance used a medium of this kind which he prepares as follows:

To 1 liter of ordinary peptone bouillon he adds 0.2 gram of trypsin, then adds chloroform and toluol to prevent bacterial growth and incubates for twenty-four hours to forty-eight hours at 37°, subsequently filtering and diluting with three parts of physiological salt solution. In the absence of conclusive data as to the advantages of this medium, the committee recommends that organisms be tested both in ordinary peptone solution and in the trypsinized bouillon of Frieber.

The second objection to indol tests, namely, the inaccuracy of methods for detecting indol has been discussed in some length by Frieber. Indol has generally been detected by the Salkowski method, that is through the use of sodium nitrite and sulphuric acid. This test has been realized for some time to be an inaccurate one and recently Frieber has shown quite conclusively that this reaction is positive not only with indol and with the methyl-indols but with indol-acetic-acid as well. The most satisfactory test for indol is now regarded to be Ehrlich's test which gives a positive reaction only with indol itself and with

$\alpha$ -methyl-indol. The Ehrlich reagent, however (p-dimethylaminobenzaldehyde), is rather too expensive to use in routine laboratory work. For this reason tests with other reagents are often preferred, as for example the Vanilin test. The Vanilin test is a very convenient one to make and gives a more nearly true indol reaction than does the Salkowski test. Frierber shows, however, that it gives a positive reaction not only with the compounds that give the Ehrlich test but also with  $\beta$ -methyl-indol.

In the light of this contribution to the subject the committee suggests the following procedure, pending further investigation of the method: Test all the cultures by the Salkowski reagent; then test the positive cultures further with the Vanilin test; and lastly test those that are positive to Vanilin with the Ehrlich reagent. Record on the chart what reaction is obtained with each test used, because it is very evident that the different reagents indicate the presence of different compounds.

The Salkowski test is made as follows: Mix 5 cc. of the culture with about one-third its volume of 1:1 sulphuric acid. Then add on the surface a small amount of a 0.02 per cent solution of sodium nitrite. A positive reaction is indicated by a pink zone between the acidified culture fluid and the nitrite solution.

The Vanilin test is as follows: To 5 cc. of the culture add 5 drops of a 5 per cent solution of vanilin in 95 per cent alcohol and 2 cc. of concentrated sulfuric acid. Indol gives a clear orange by this test which reaches its greatest depth in two or three minutes. Tryptophane, on the other hand, gives a reddish violet which develops more slowly and deepens on standing or heating.

The Ehrlich test is made as follows: The reagent is a 2 per cent solution of para-dimethylaminobenzaldehyde in 95 per cent alcohol. One cubic centimeter of this reagent is added to the culture, then drop by drop concentrated hydrochloric acid is added until a red zone appears between the alcohol and peptone solution. Not more than 0.5 cc. of the acid is required. On standing the zone deepens and widens. The red color is soluble in chloroform and the test may be confirmed by shaking the culture with chloroform to see if the pigment dissolves. If it proves soluble the test is considered positive.

#### PRODUCTION OF HYDROGEN SULFIDE

The method of determining hydrogen sulfide which seems to be in most common use is the lead acetate agar method. This

method was described by Kligler (1917) and has been reported upon favorably from other sources. Although certain weaknesses in the technic have been pointed out it is given here as a provisional method. The committee has not yet had an opportunity to give it the investigation it needs and therefore does not indorse the method.

Prepare an agar like the standard peptone-beef-extract formula but with 30 grams instead of 5 grams of peptone per liter, and with a reaction between  $\text{pH} = 7.2$  and  $\text{pH} = 7.6$  (i.e., just at the alkaline, or blue end of the sensitive range of brom thymol blue). Sterilize this medium 5 cc. to the tube. Sterilize separately a 0.1 per cent solution of basic lead acetate, and after sterilization add 5 cc. to each tube of agar, when the agar has cooled to about  $50^{\circ}\text{C}$ . Allow the tubes to cool in an upright position, and incubate for a long enough period to be sure that no contamination is present. Inoculate these tubes with the organisms to be tested by stabbing with an inoculating needle. Incubate at optimum temperature for from eighteen hours to a few days according to the vigor of the culture. Hydrogen sulfide causes a blackening or browning of the medium along the line of inoculation.

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# AN INVESTIGATION OF AMERICAN GENTIAN VIOLETS

## REPORT OF COMMITTEE ON BACTERIOLOGICAL TECHNIC

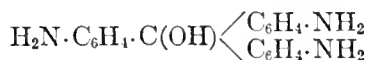
PREPARED BY H. J. CONN, CHAIRMAN

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This report is a continuation of the work on American stains that has recently been published in this journal (Conn, 1922). In this earlier report fairly definite conclusions were reached in regard to fuchsin and methylen blue but the work on gentian violet was regarded as being unfinished.

Gentian violet is a term which does not refer to any definite chemical compound, but rather to a mixture of dyes of a certain group. The term was apparently originated by Grüber and is not recognized in the dye industry or in the textile industry. The dyes in the mixtures sold as gentian violet all belong to the pararosanilin series but at the present time the different manufacturers and dealers nearly all sell different mixtures of these dyes under the name of gentian violet. This being the situation, the best use for the term gentian violet seems to be as a generic designation for any violet pararosanilin which has the general staining properties recognized as belonging to Grüber's gentian violet.

As mentioned in the earlier report the pararosanilin base has the formula



The six hydrogen atoms of this formula which are in the amino-groups can be replaced by a methyl group, an ethyl group or a benzyl group. The most commonly known compounds of the pararosanilin series contain in these positions 4, 5, and 6 methyl groups respectively, and are known as the tetramethyl, penta-

methyl and hexamethyl pararosanilins. The mixtures of lower methylation have the most reddish cast of all the dyes in the series and are generally designated as methyl violet. As the mixture contains larger and larger amounts of more highly methylated compounds, it becomes bluer in color and the successive deeper shades are known in the textile trade as methyl violet B, methyl violet 2B, methyl violet 3B, etc. Those compounds designated as methyl violet 5B, 6B, and 7B, respectively, are still bluer in shade and apparently contain a pararosanilin in which a benzyl group has been introduced into one of the amino-groups.

The only textile dye in this series which if pure has a definite chemical formula is crystal violet, which is hexamethyl pararosanilin. This dye is quite a deep blue violet.

In the earlier work already reported a comparison was made of various commercial samples of methyl violet, methyl violet B, methyl violet 2B, methyl violet 6B and crystal violet, which were compared with certain samples obtained from different biological supply houses labeled as gentian violet. It was found that crystal violet and methyl violet 6B were at least fair substitutes for gentian violet in the Gram stain but that the methyl violets of lower methylation were unsatisfactory for this purpose. The present investigation was planned as a more intensive study of crystal violet and methyl violet 6B in comparison with gentian violet.

In the second series of tests there were included 10 samples of crystal violet, 8 samples of methyl violet 6B, and 10 samples of gentian violet. Of these 28 samples, 19 had been included in the earlier work.

Two of the samples of crystal violet included in the earlier work, namely those from the Providence Chemical Company, and from Dicks David and Company, unfortunately had to be omitted from the second test, due to the exhaustion of the samples that had been investigated; but on the other hand in the present work three samples of crystal violet, four of methyl violet 6B, and two of gentian violet were added which were not included in the first tests. Of these 9 additional samples, three were

Grübler's, but they were distributed to the collaborators designated by number only, and were in most cases thought to be American samples.

The collaborators were instructed to use these samples of stain in staining bacteria with the Gram technic. To make the results more constant if possible than in the earlier work, directions for making the tests were furnished and cultures were distributed for use as test organisms. The cultures furnished were: *Bacillus cereus*, as a strongly gram-positive organism; a small short rod of the fluorescent group, as a decidedly gram-negative organism; and lastly an unidentified micrococcus which had been found to be weakly Gram-positive although often negative with poor technic. The directions for making the stains which were furnished were as follows:

Twenty-four hour cultures should be used in the test, the coccus incubated at 37°, the other two at room temperature (or 25° if available).

To make results strictly comparable the procedure should be carefully controlled and timed as follows:

Stain thirty seconds.

Drain but do not wash.

Iodine thirty seconds.

Drain but do not wash.

Ninety-five per cent alcohol until no more stain is removed; not over two minutes.

Keep in agitation, and transfer without washing to

Counterstain thirty seconds.

Wash and dry.

*Stain formulae*

The gentian, crystal or methyl violet may be made up according to one or both of the following formulae. If only one formula is used, select formula A; but if convenient it is hoped that formula B may also be used for the purpose of comparison.

<i>A. Regular formula</i>		<i>B. Optional formula</i>	
	<i>cc.</i>		<i>cc.</i>
Saturated alcohol solution of stain	6	Saturated alcohol solution of stain	10
95 per cent alcohol	5	1 per cent ammonium oxalate solution	40
Anilin water (1:49, filtered)	50		

Lugol's iodine solution should be used (1 iodine: 2 KI : 300 water.)  
The counter-stain may be selected according to personal preference.

Basic fuchsin . . . . . (1:10)  
Safranin . . . . . (1:10)  
Eosin . . . . . (saturated alcohol)

Bismark brown (2 per cent) may be used, but the staining period should be longer.

In making reports be sure to specify the counter-stain used.

Of these two formulae the one containing anilin (A) was selected because in a series of preliminary tests made with 4 different samples of gentian violet and using 20 different technics, this particular formula was among the best if not the very best. The ammonium oxalate formula (B), however, proved almost if not quite as good in these preliminary tests and the solution keeps indefinitely. It was such a new formula, however, that it did not seem best to include it in the present series of tests as a regular formula; hence formula (A) was selected for this purpose with the ammonium oxalate method as an optional formula, in order to get some information as to how well the latter compared with the anilin formula. Five investigators, it will be seen, used formula B and obtained as good results with it as with formula A.

The results of the tests are given in table I. In this table the same method of indicating results is used as was employed in the earlier article. The significance of the symbols used is as follows:

E, excellent  
G, good  
F, fair  
U, unsatisfactory

One column of this table indicates the average rank of the first tests, that is those reported in the previous paper, and another column indicates the average rank in the present series of tests.

The first thing to be noticed in the present series of tests is that all the samples have nearly the same rank in their average grade, which is generally G, G + or G -. Provided all the results





are averaged, there is very little choice between crystal violet, methyl violet 6B, and gentian violet. A few more F grades appear with crystal violet than with either of the others but not enough to be of any significance. For our practical purposes these results indicate that the Gram stain can be made with a dye bearing any one of these three names, provided it comes from a satisfactory source.

Quite in contrast to the uniformity of these average results is the variation in the reports from the individual investigators. In spite of the standardized technic used in the work there is not a single sample in regard to which entirely consistent results have been made and practically every sample that has been tested more than four or five times has been reported good or excellent by some investigator and unsatisfactory by some other. This suggests that either the personal equation is one that cannot be eliminated entirely or else that some point in the technic requires further standardization. The chief points which have been mentioned so far as susceptible to further standardization are: temperature at which the cultures are incubated, strength of alcohol used for decolorization, length of time of decolorization, and nature of the counter-stain used. It is very doubtful whether variation could be avoided by controlling all these factors but the committee hopes to plan a further investigation along this line simply to test out some of these points in regard to the Gram technic. In a recent paper Burke (1922) has suggested the importance of some of these factors, but it seems as though a coöperative experiment might give more light on the subject than any study made by a single observer.

In looking up the results of the individual manufacturers of these stains, it is to be noticed first that nearly every sample which has ranked as high as G+ or E- is one that has been tested so few times in this work that the results are not especially significant. For this reason it is regarded as impossible to decide which is the best of the samples tested. It is perhaps of some significance, however, to notice those few samples that have ranked below G- in the general average. These samples are crystal violet of Harmer, H. S. Laboratories, Leitz and

Grübler, the methyl violet 6B of Providence, and the gentian violet of Harmer. With the exception of these few samples there seems no question but that the rest are entirely satisfactory for the Gram stain, and the American samples have ranked practically as well as the Grübler samples. In fact, the Grübler crystal violet ranks the lowest of any of the samples tested.

The chief conclusion from this work seems to be that samples of these three dyes are now produced in this country which are perfectly satisfactory for the Gram stain, apparently just as good as the Grübler samples, and that either crystal violet or methyl violet 6B can be substituted for gentian violet in the gram stain. The committee especially recommends the use of crystal violet for this purpose as it is the one dye of this series for which a definite chemical formula can be indicated, although commercial samples of it are not necessarily pure and may vary in their composition. This variation is probably less than in the case of methyl violet 6B, where the name denotes merely a certain shade of violet, and in the case of gentian violet, where the name is not used in the dye industry and is not mentioned in Schultz's index of dyes. For this reason the committee recommends that those ordering stains to be used in the Gram technic should specify crystal violet instead of gentian violet.

Turning to the results with the different samples of crystal violet, it will be seen that only one of those investigated was from a basic manufacturer, namely the Du Pont sample. This sample and the Goldin sample have given the highest average grade of any of the crystal violets tested. All of the other crystal violets seem to fall slightly below the Du Pont sample which is rather surprising because there are indications to suggest that nearly all of these dealers in biological stains merely rebottle the Du Pont crystal violet and sell it under their own name. If this is the case, the slightly higher rank of the Du Pont sample may be considered to be pure accident.

The only other actual manufacturer of crystal violet in this country of which we have knowledge is the National Anilin and Chemical Company. Their product was not included in

this work but there is reason to believe that it is carefully prepared and cursory examination of slides stained with it by the gram technic indicates that it is as satisfactory as the Du Pont product. In this connection it should be remarked that the Du Pont Company does not sell crystal violet except in bulk, whereas the National Anilin Company is now making a speciality of biological stains and sells them in small containers. If the Du Pont product is desired, it should be ordered through some supply house that can be trusted to supply the actual products specified.

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C.-E. A. WINSLOW



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## CONTENTS

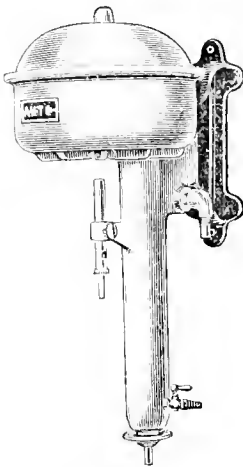
J. ØRSKOV. Method for the Isolation of Bacteria in Pure Culture from Single Cells and Procedure for the Direct Tracing of Bacterial Growth on a Solid Medium . . . . .	537
WILLIAM S. STURGES AND LEO F. RETTGER. Bacterial Autolysis . . . . .	551
T. M. RIVERS. <i>Bacillus hemoglobinophilus canis</i> (Friedberger). ( <i>Hemophilus canis</i> Emend) . . . . .	579
J. M. SHERMAN, G. E. HOLM AND W. R. ALBUS. Salt Effects in Bacterial Growth. III. Salt Effects in Relation to the Lag Period and Velocity of Growth . . . . .	583
LEON S. MEDALIA. Further Observations on "Color Standards" for the Colorimetric Determination of H-Ion Concentration . . . . .	589
JOHN WEINZIRL. The Cause of Explosion in Chocolate Candies . . . . .	599
SELMAN A. WAKSMAN. Microorganisms Concerned in the Oxidation of Sulfur in the Soil. IV. A Solid Medium for the Isolation and Cultivation of <i>Thiobacillus thiooxidans</i> . . . . .	605
SELMAN A. WAKSMAN. Microorganisms Concerned in the Oxidation of Sulfur in the Soil. V. Bacteria Oxidizing Sulfur Under Acid and Alkaline Conditions . . . . .	609

Abstracts of American and foreign bacteriological literature appear in a separate journal, *Abstracts of Bacteriology*, published monthly by the Williams & Wilkins Company, under the editorial direction of the Society of American Bacteriologists. Back volumes can be furnished in sets consisting of Volumes I, II, III and IV. Price per set, net, postpaid, \$24.00, United States, Mexico, Cuba; \$25.00, Canada; \$26.00, other countries. Subscriptions are in order for Volume V, 1921. Price, per volume, \$5.00, United States, Mexico, Cuba; \$5.25, Canada; \$5.50, other countries.

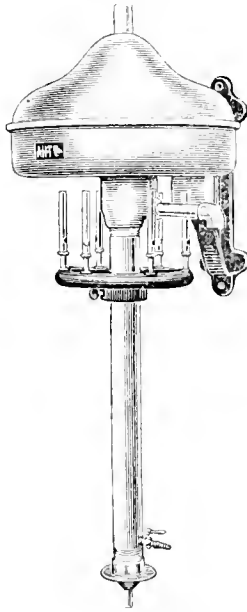
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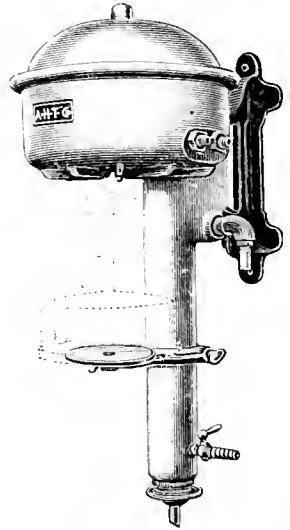
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# METHOD FOR THE ISOLATION OF BACTERIA IN PURE CULTURE FROM SINGLE CELLS AND PROCEDURE FOR THE DIRECT TRACING OF BACTERIAL GROWTH ON A SOLID MEDIUM

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By a pure culture we understand, as is well-known, a culture consisting of individuals of which we know with certainty that all are descended from one single cell, and from one only. As all bacteriologic work, of whatever kind it may be, depends on our working with such reliable pure cultures, many efforts have, of course, been made in the course of time in order to devise reliable methods of isolating a single bacterium.

The first investigator who solved the problem in a satisfactory way,—although not in regard to the bacteria proper—was Emil Chr. Hansen. The principle of his method was, briefly stated, to observe directly under the microscope the growth of the individual yeast-cell until it has formed a small colony in a gelatin droplet on the lower surface of a coverglass in a moist chamber. Yeast-cells are however far bigger than most bacteria, and there is no possibility of tracing with any certainty the growth of a bacterium, as for instance a colon bacillus, in a similar way in gelatin.

Of methods that have been proposed and employed for single cell cultivation of bacteria, the best known are those of Schouten, Barber and Malone, none of which have however attained any extensive application, no doubt partly owing to the intricate apparatus they require, and partly to the difficulty involved in picking up such minute objects as bacteria with such relatively coarse implements as pipettes and loops; and when Barber states that he is able to pick up successively each single one of four

bacteria, which he views in a small hanging-drop of broth, and to inoculate four broth test tubes with each of them separately, one feels predisposed to doubt the possibility of ever acquiring such practice.

The method most generally used at present is, no doubt, Burri's India ink method, which is the starting point for the procedure described in the following communication. As is well known, the principles of the India ink method are, briefly stated, these: the bacteria are emulsified in diluted India ink, of which emulsion minute droplets are deposited on a gelatin plate in a Petri dish by means of a mapping-pen. Those droplets which, by microscopical investigation with a high power dry lens, prove to contain only one single cell, are noted and allowed to stand until a small colony has developed, from which subcultures are prepared; or, the India ink droplet is removed, together with the bacterium, by means of a sterile coverslip that is superimposed on the black spot of the gelatin plate, removed again together with the India ink and the bacterium, and dropped into an appropriate fluid nutrient medium. This is, as has been said, an excellent method, by means of which, with some practice and patience, reliable single-cell cultures of most species of bacteria can fairly easily be produced. (It is, however, not all bacterial species that will stand the India ink.)

A drawback in Burri's method is the necessity of having the unhandy Petri dish standing on the microscope stage during the examination. Therefore, I devised a modification: by means of sterile Pasteur pipettes I poured liquid gelatin upon previously sterilized slides. On the gelatin surface three rows of India ink droplets were deposited, which could now be much more easily and rapidly examined by shifting the mechanical stage, the selected India ink droplets being subsequently removed as usual by means of sterile coverslips.

If it is desired to trace the development of the new formed elements on the gelatin, the slides are placed in a sterile Petri dish with a piece of moist filter paper at the bottom. In this way the India ink spot can be examined at intervals, and the development can be observed. The image however, will rapidly

become blurred under such conditions. The India ink will be broken up, the new-formed elements pushing in beneath it; even though the India ink be highly diluted, which facilitates observation, the image will rapidly lose its sharpness. Our objective being, in my case, partly to obtain single-cell cultures from some atypical bacterial elements, and partly to trace their development, it seemed natural, to attempt to do completely without the India ink, the multiplication of the bacteria being much easier to follow, the more dilute the India ink. When this was done I admit being surprised at seeing how well the organisms showed up as sharply defined, highly refractive elements, readily distinguishable from other chance particles or impurities on the surface.

Gelatin is however not a particularly suitable medium for most bacteria, and growth at 37°C. could not be observed in this manner, so I tried whether the bacteria were as clearly visible on an agar surface. In order to study this point, agar (common filtered broth agar) was melted, and by means of a coarse Pasteur pipette poured over a sterile slide. The bacteria were even more readily discernible on this surface than on the gelatin. It is difficult however to procure a perfectly level agar surface in this manner. This difficulty was overcome by abandoning the pouring of the agar on the slide and, instead, excising the medium out of an agar plate in a Petri dish by means of a knife, previously sterilized in a flame and cooled, lifting the excised cube of agar on the blade of the knife and depositing it on the sterilized slide to which it will immediately adhere.

The essential conditions were now provided for tracing bacterial growth on a solid medium, especially if certain difficulties could be overcome in regard to ensuring reliable and readily obtained single-cell cultures. At this point in my investigation a paper appeared in *The Journal of Hygiene* by Hort, in which he describes a method, the underlying principles of which are the same, namely, the observation of unstained bacteria without a contrast on an agar surface, partly by means of oil-immersion lenses, partly by a system of high-power dry lenses. Having given a review of the usually employed methods of isolation,

all of which, including Burri's method, he rejects, he suggests a new method of isolation having two modifications, one in which examination is undertaken with an oil-immersion lens, and one in which a dry lens is employed.

In bacteriologic literature, Hort is thus the first to point out that it is possible to see bacteria distinctly on the surface of an agar plate and to watch their growth by means of a high-power system of dry lenses without any staining or contrast. It is true that Hill, in his work on the morphology of the diphtheria bacillus, mentions that diphtheria bacilli can be seen distinctly on agar by means of a high-power dry lens, but he does not seem to realize the possibilities involved in this fact. It is no doubt possible, and I think, probable, that others too have been aware of this fact; Hort is however, as stated, the first who has defined it and understood how to make use of it.

The medium which Hort employs in his examination, he prepares in a manner similar to the one originally applied by me. He pours the hot agar over sterile slides in as level a layer as possible, taking care to keep the agar well within the edges of the slide. The mode of procedure now varies as to whether he employs the oil-immersion lens, or the dry lens system for further examination. In the first case a series of sterile coverslips have been previously prepared, each with a small circle etched on its surface by means of a diamond. In the center of this circle, a minute droplet of broth is now deposited, taken from a broth culture containing the bacteria under investigation in a suitable emulsion. The inoculated coverslip is now placed face downwards on the agar surface; the area within the small circle is thoroughly examined with an oil-immersion lens, and, in case only one single organism is found within the circle, the slide is placed in a Petri dish which is then placed in the incubator. The circle is examined at short intervals, until a small colony has formed from which subcultures can be prepared.

Hort himself remarks, in regard to this procedure, that great care must be taken to ensure that the droplet does not run outside the etched circle when the coverslip is applied to the agar, adding, however, that with some care this is easily avoided. It

is evident that we must feel perfectly sure on this point, considering that the area within the circle is the only one examined. However, it seems difficult to understand how one can be sure that the small droplet keeps within the etched circle, as a small liquid layer will form between the coverslip and the agar, the moment these two are directly applied to each other, which will immediately make the droplet invisible. The possibility will likewise always exist, of various currents arising between coverslip and agar, both when the slip is placed and removed; and, even though the growth of a colony from one single cell has been observed within the circle, this may become contaminated from a small colony immediately outside the circle, the moment the coverslip is removed. As a rule, there is little chance of this happening, but it does compromise the reliability of the method.

Moreover, there is the question of the power of certain motile bacteria to move on the uncovered agar plate. The possibility of such active motility on the part of the organisms is, of course, increased by the placing of the coverslip on the agar, by which a small liquid-filled space is formed.

For his second method Hort employs a medium prepared in a similar way. A highly diluted emulsion of bacteria is spread over the agar plate by means of a glass rod, the inoculated plate being now covered with a thin sterile strip of celluloid, which has been previously perforated. Small sterile coverslips are placed over the holes so as to form minute moist chambers. These are now searched with a high-power dry lens and the chambers containing one single cell only, are marked. The examination is now continued as described above. (It is not evident from Hort's treatise, whether the coverslips are removed during the examination under the microscope; if not, one would think that the dew on the coverslip would be obstructive.) The colonies having reached an appropriate size, subcultures are prepared by means of a special apparatus consisting of a tube with a needle adjusted in a special way and screwed on to the nose-piece like an objective. This method is reliable, but as Hort himself says in his final remarks: "In conclusion it is necessary to point out that cultivation of bacteria from single cells is, even when

employing a good method, a most tedious procedure, involving several hours' close work for each organism isolated, if the results are to be relied on."

I believe the difficulties to have become considerably reduced in the procedure described in the following pages, and, the simpler a method is, the more reliable will it generally be. The principle upon which the method of isolation described below has been based is, as mentioned, the fact that a bacterium—possibly the minutest ones excepted—can readily be distinguished on the surface of a clear transparent medium, such as for instance agar, gelatin, or ascitic agar. The mode of procedure is, briefly stated, as follows: A young bacterial culture, such for instance as a twelve-hour old broth culture of colon bacilli, is inoculated on the agar plate in a Petri dish. The agar had better not be more than a few millimeters thick (bacteria can also be distinguished on very thick agar, but less sharply). The upper and lower surfaces of the agar should be parallel so as to ensure that the excised bits shall be of equal thickness everywhere, partly in order to obtain plane images, partly to avoid the risk of running down with the objective into the agar, which is in its immediate vicinity during examination.

It is important, in inoculating the culture, to be fairly clear at the outset as to the density of bacteria desired on the plate. In the diagram, figure 1, some dotted lines show how I am accustomed to proceed. A big droplet of the broth culture is deposited in the center of the circle, and, by means of a glass rod, bent at a right angle, the drop is pressed down between the parallel dotted lines. Now the glass rod is moved from side to side across the first inoculated area, and, finally, the remaining half of the dish is inoculated and it is placed in the incubator for about one hour at 37°C. (Inoculation can of course also be performed in a streak, which some will perhaps find more to the purpose, and in this way an appropriate difference in the density of the bacteria can likewise be obtained.) This measure is taken because the development in the case of the colon bacillus begins just after the expiration of one hour, and because bacteria are more readily discernible when in development, owing



to their increased refractive power. As previously described, a suitable square of the agar is now excised and placed upon the previously sterilized microscope slide, which is most conveniently sterilized by flaming (cf. fig. 2).

The microscope slide plus agar, which, for convenience sake, I shall term "slide" in the following discussion, is placed on the stage of the microscope, and an area is chosen where the organisms are placed at a convenient mutual distance, commencing

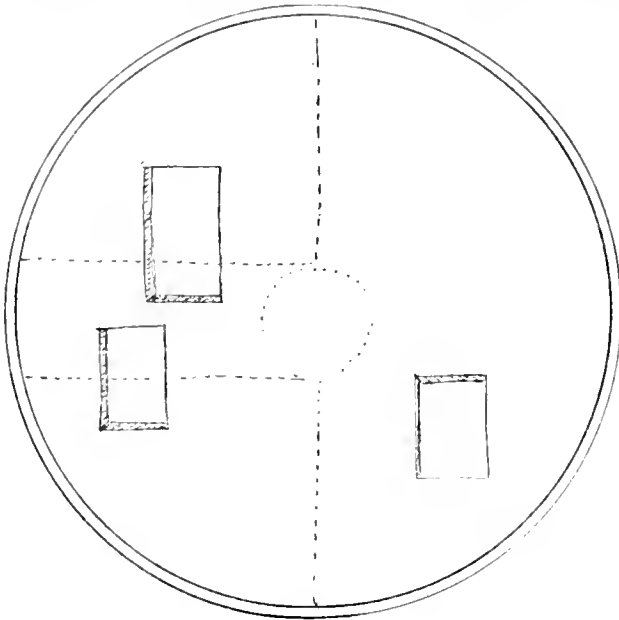


FIG. 1

the examination where they are lying close, and thence, by means of the mechanical stage, proceeding to where they are lying more scattered.<sup>1</sup>

Having now come upon an area where there is one organism, only, within the field of vision, and this single bacterium having

<sup>1</sup> The objective employed by me was a Zeiss Apochromat; any sufficiently powerful objective will however do. The magnification, at which I usually worked, was 750 diameters. Illumination is a very important factor. The source of light must be uniform. I used a powerful metal filament lamp with frosted bulb, the light of which was considerably reduced by means of the diaphragm of the illuminating apparatus of the microscope.

been centered, the area is noted by means of the mutual relation of the regular scale and the vernier attached to the mechanical stage, and one should now be able to focus exactly in the same place again. At this point there is however a great difficulty to be overcome, as the least inaccuracy in the re-adjustment may have the effect of causing the selected organism to vanish from the field of vision, others being substituted, and,

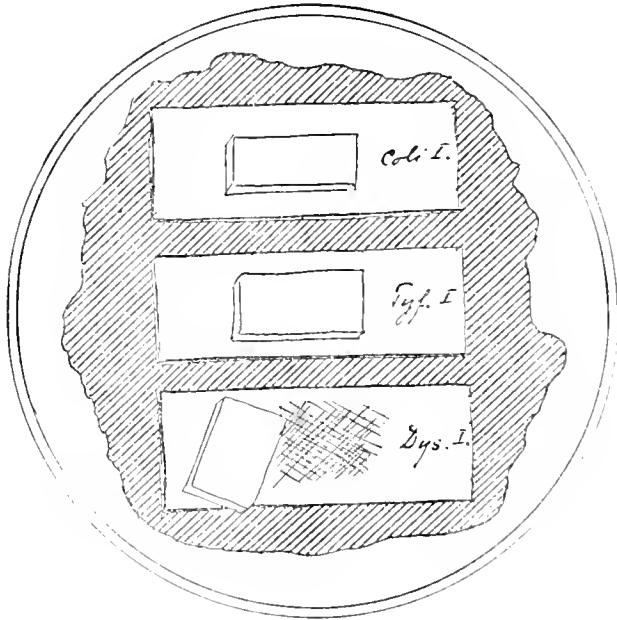


FIG. 2

even though the most painful care be taken, the same fatal accident may happen owing to quite negligible displacements of the scales, which it is often quite impossible to control.

In order to be able to find a particular bacterium again, I have proceeded in the following manner: Prior to placing the agar plate on the slide, a complex of fine lines are by means of a diamond scratched criss-cross, preferably on the lower surface of the slide, over the area to be covered by the agar (cf. fig. 2, above). The lines will become less frayed in the glass if the

scratching be performed in a drop of immersion-oil. In an objective (preferably a different one from that with which the growth is observed, as the micrometer lines will disturb observation) is placed a squared eyepiece micrometer, which is cemented on to prevent displacement.

If we now focus sharply on the scratches on the slide with the low power of the microscope, the lines in the eyepiece micrometer will be intersected by these scratches in a quite specific manner (cf. fig. 3), and thus we obtain two distinguishing marks instead of one. The course of procedure will now be as follows: the agar is placed on the scratched area of the slide, and we search for a place where there is only one organism within the field of vision. The spot is marked by means of the scales of the mechanical stage, the objective with the attached micrometer is placed in the tube, the microscope is adjusted to low magnification and focussed sharply on the scratches. Careful drawings are made on squared paper of the position of these scratches in relation to the eyepiece micrometer, the slide being now placed in a sterile Petri dish with a piece of moist filter paper at the bottom. (The filter paper must not be too wet as this may cause the development of so much aqueous vapour during incubation that the glasses become wet enough for the agar squares to slide, when the whole experiment is ruined.) By means of thus marking the position of the organism we have always succeeded in hitting upon exactly the same spot for repeated examination.

The growth is now watched at proper intervals, the adjustment being performed so that, firstly, the scales of the mechanical stage are placed in the proper mutual relation, which we have noted down, secondly, with the low power of the microscope we make the scratches on the slide correspond to the proper points in the eyepiece micrometer, and, finally, eyepiece and objective are changed, and we can now easily observe the alterations in the small colony in development. (The slides must of course previously be cooled to the same temperature as that of the objective, in case examination takes place at a lower temperature than that of incubation, as, otherwise, condensed moisture

will gather on the front lens and obstruct vision altogether.) In this way, the agar will preserve its shape for at least twenty-four hours, even though subjected to several examinations, provided these be not of too long duration.

Now, do not these repeated examinations involve a great danger of contamination from the air? A risk there is, of course, but it is apparently, insignificant. In the first place it can easily be ascertained that only very few "alien" colonies will be seen

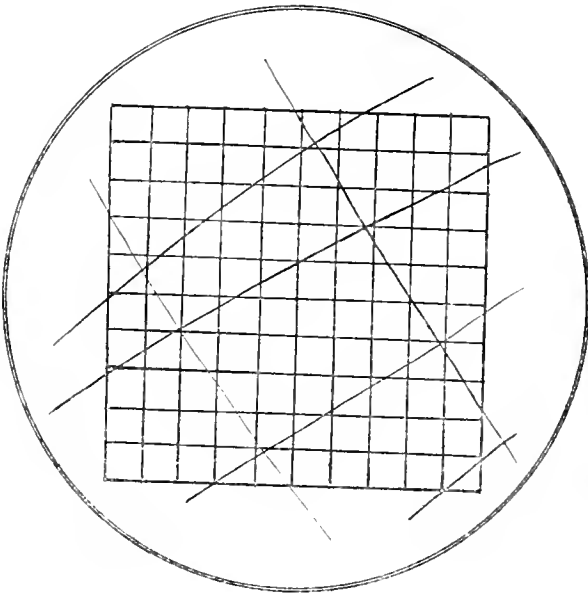


FIG. 3



FIG. 4

to develop in the Petri dish by contamination from the air, in spite of repeated examinations within forty-eight hours, and it would be a stroke of very bad fortune if such a germ from the air should settle just within the field of vision to be examined. If this should occur, it would soon be discovered; it could only escape detection in case a germ from the air dropped upon the selected colony immediately before inoculation was undertaken from it, and then it would most probably be disclosed in further investigations of the bacterial species in question, as it would

be almost inconceivable ill-luck if the "alien" microörganism should be one that was closely related to the isolated one.

Now, the new-formed colony having reached a convenient size, sub-cultures should be prepared from it. At this point the colonies have grown so large as to be distinctly visible with the low power of the microscope, the time and mode of re-inoculation depending on the relative situation of the colonies. In case the colony, the shape of which we recognize with the low power of the microscope, is placed in a sufficiently isolated position, we may defer inoculation until it has grown big enough to allow of our conveniently inoculating from it by means of a fine inoculation needle under the microscope at low magnification. If there is any danger of neighbouring colonies impinging upon it, we must undertake inoculation while it is yet small. As mentioned, Hort used a special apparatus for this purpose. A small harpoon, which one may prepare oneself, will however do.

On the front lens of an objective is placed a small lump of modelling wax to which is attached a fine thin platinum wire not thicker than 0.15 mm. with a blunt end (cf. fig. 4). Previous to "harpooning" the colony some preparatory practice is necessary. A small agar square is excised and fitted in the usual way and placed on a slide. A droplet of India ink or some other staining fluid is deposited on the agar with a mapping-pen. This spot is now centered in the field of vision at the low power of the microscope, and the objective with the attached platinum needle, which is screwed on to the nose-piece, is directed across the spot, the needle being adjusted by a pressure from the fingers so as to be mounted exactly above the spot, and gently depressed so as to touch the agar. The point of contact is readily discerned with the low power of the microscope and marked by means of the eyepiece micrometer. We know now exactly where the needle will hit, being able then to inoculate from the colony by adjusting it to the exact point in relation to the eyepiece micrometer at which the needle hit last. The needle is carefully lowered into the colony until it touches the agar plate. The point of contact is most readily noticed by

following the reflection of the needle in the agar; when the needle and its image meet, contact with the colony has been established. The point of the needle is now touched with broth in a small loop which is raised so as to encompass it several times, agar is inoculated from the loop, and finally the needle is washed in a tube of broth.

The "harpoon" is sterilized by flaming, and now it only remains to examine the area where the colony was previously situated. The bacteria from the colony will be seen to have been scattered somewhat, and we note whether the adjacent colonies are totally intact, both with the low and with the high power of the microscope. In case growth results from the inoculation we know that we have obtained a reliable pure culture. Compared with Hort's dry-lens method, the procedure described presents several advantages. Firstly, it is difficult to pour agar over the slides so as to obtain an even layer, and it takes a long time. Petri dishes with agar are always at hand in any bacteriologic laboratory; these should however be freshly poured to avoid the risk of obtaining pure cultures from chance microorganisms from the air which, being overlooked at inoculation, may have formed small colonies. Secondly, we avoid the perforated celluloid plate which is a hindrance to free operation and means a considerable limitation in the possibility of finding conveniently placed microorganisms. While Hort spends several hours on the pure cultivation of a bacterium by his method, I believe that the total work in my isolation method will, in most cases, only amount to a fraction of an hour for each single bacterium.

As has been shown, we are able to trace the growth from the single cell until a small colony has developed. Details can of course only be observed as long as the colonies are small and single-layered; so soon as the colonies are crowded in several layers, exact examination is of course out of the question. If, for instance, we desire to ascertain whether a morphologically atypical element is viable, and to follow its development, we isolate it as described above and observe its growth at proper intervals. If it is desired to get a survey of the way in which colony formation proceeds we need only, at proper intervals,

to excise small bits of the agar plate inoculated with a bacterial culture, and we obtain in this way a far better picture of the actual morphology of the bacterium than by producing preparations according to the usual methods, whether it be the milder procedure of emulsifying the bacteria in a drop of a staining fluid, or one of the various staining methods with previous fixation.

If, owing to the minuteness or too crowded placing of the microorganisms, we should fail in distinguishing what we desired to see, such as for instance the cell division lines, by means of the dry lens system, we need only place a coverglass on the surface of the agar. This will immediately adhere to the agar, and, by means of the oil-immersion lens we shall be able to detect the bacteria quite distinctly and also to trace, the growth of a single element.

Any one can readily be persuaded as to the facility with which bacteria are distinguished on an agar surface without staining or contrast, by examining an inoculated agar plate after a few hours' incubation.

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## BACTERIAL AUTOLYSIS

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The term "autolysis" has quite generally been used to designate a process of cell or tissue deterioration in which the more complex nitrogenous substances, particularly proteins, are reduced to simpler form through the agency of enzymes which have been elaborated by the cells or tissues themselves.

The self-digestion of yeast, and of liver and other animal organs was recognized by Salkowski (1891) to be due to enzymatic action. An increase in the soluble nitrogenous constituents and the presence of leucine and tyrosine were observed. Jacoby (1900) showed that there is a consistent increase in ammonia during the process and advanced the hypothesis that this constituted the mechanism for the elimination of soluble katabolic products of the normal living cell.

Fermi (1890, 1891 and 1894) was among the first to demonstrate the presence of enzymes in bacteria. Extensive comparative studies with pepsin and trypsin showed that the proteolytic principles of bacteria resembled the latter. Our knowledge of the proteolytic enzymes of bacteria was further advanced by Wood (1890), MacFadyen (1892), Vignal (1896), Eijkmann (1901) and Cacace (1901).

By the employment of Buchner's "Hefepressaft" method Hahn (1900) obtained an extract from *Bact. typhosum* and *Mycobact. tuberculosis* which he claimed possessed autolytic properties. Brieger and Mayer (1903 and 1904) reported that they had removed agglutinogens and other specific bodies from *Bact. typhosum* by autolysis. It appears quite probable, however, that the processes which they employed were such

that osmosis cannot be excluded as an important factor. Conradi (1903) described an "autolysis" of *Bact. typhosum* and *Bact. dysenteriae* whereby endotoxins were liberated. He believed that all bacteria possess autolytic enzymes. Neisser and Shiga (1903) reported similar observations. They found, however, that the sterile filtrates of twenty-four hour cultures which had been heated at 60°C. for one hour and kept at 37° for two days showed the presence of free "receptors" which had the power of absorbing agglutinins and depressing agglutination. The filtrate from the dysentery bacillus culture was as toxic as that obtained by Conradi. Hence, in view of this heating, one is not justified in saying that the toxin is liberated by autolysis.

The autolysis of *B. anthracis* was described by Levy and Phersdorff (1902). Microscopic changes were noted in alkaline suspensions to which toluol had been added. Gelatin-liquefying and other enzymes were observed, and the authors claimed that the autolysate was toxic to white mice. However, as much as one thirteenth of the body weight had to be injected in order to obtain these toxicity results.

Rettger (1904) applied chemical criteria to the study of the autolysis of microorganisms and pointed out qualitative changes of considerable significance. He noted the liberation of coagulable protein and subsequently the formation of leucine and tyrosine in water suspensions of *Erythrobacillus prodigiosus*. The biuret test was found to be very valuable in following the course of autolysis. Microscopic changes in stained mounts were followed also. During the incubation of water suspensions of *Bact. coli* to which toluol had been added there was apparently some liberation of coagulable proteins from the cells. It is impossible to state whether this was the result of real autolysis or of osmotic changes.

In Flexner's study of the meningococcus (1907) the interesting observation was made that this organism resists disintegration longer when kept at 37°C. than in the refrigerator. Concentrated suspensions in physiological saline solution were found by microscopic examination to undergo rapid autolysis. Wollstein (1907) showed that the meningococcus and gonococcus

were very much alike in their behavior under the various experimental conditions.

Bürgers, Schermann and Schreiber (1911) claim to have observed autolysis by *Bact. coli*, *Bact. typhosum* and the pneumococcus after killing with chloroform. According to them, however, *B. megatherium*, Staphylococcus and Streptococcus do not autolyze. McClintock and Clark (1909) satisfied themselves that the rapid lysis which suspensions of the gonococcus undergo is due to enzymatic activities. Heating at 70°C. for one hour prevented the cellular changes from taking place. Rosenow (1912) maintained that substances capable of causing anaphylaxis are liberated by the pneumococcus, streptococcus, gonococcus, meningococcus, *Bact. coli*, *Bact. typhosum*, and to some extent by the staphylococcus.

Warden (1913, 1915, and 1917) made a rather extensive study of the autolysis of the gonococcus. In his earlier work he looked in vain for any external factors which might be responsible for the disintegration of the cells, but did observe an "enzyme," tryptic in nature, which was always present in autolyzing suspensions. In a later paper (1915), however, he concludes that "lysis of gonococci. . . is probably due, not to activities of enzymes, but to other causes, among which water permeability and solution of fatty substances play an important part."

Alilaire (1913), Nicolle (1913) and Salimbeni (1913) studied the process of so-called "autolysis" by determining the "soluble" and "insoluble nitrogen." They claim to have observed increases in "soluble nitrogen" during the autolysis of *Bact. coli*, *Bact. typhosum*, *Proteus vulgaris*, *Pseudomonas pyocyanea*, the gonococcus, meningococcus and pneumococcus. Corper (1916) and Corper and Sweeny (1918) demonstrated that suspensions of the tubercle bacillus in physiological saline solution, with or without the addition of antiseptics, undergo autolysis at 37°C., as evidenced by marked increases in non-coagulable and amino nitrogen.

Dernby (1917 and 1918) working with yeast, and later with animal tissues, was able to show the presence of various proteolytic enzymes having specific hydrogen ion concentration re-

quirements. A pepsin-like enzyme was observed whose activity depended upon a hydrogen ion concentration greater than pH 5.5. In every instance there were also present enzymes of a tryptic nature, most active in a fairly alkaline medium. The acid end of the range of the latter, however, overlapped the alkaline end of the range of the former ferments and a digestion (or autolysis) carried on in the pH zone common to both tended to be much more complete than one carried out in the alkaline range so often chosen for such experiments.

Our knowledge of bacterial autolysis is as yet far from complete, and the little information which has been acquired is confined, with very few exceptions, to the changes which take place in the protein of the bacterial cell. In some instances the investigations have not gone beyond a morphological study, and in very few cases were the changes determined by modern chemical methods.

There is considerable difference of opinion in regard to the definition of the term "autolysis." The word has been variously used to express changes in gross and microscopic appearance, loss of vitality, changes in solubility and actual changes in chemical composition. From a careful consideration of the phenomena which Salkowski originally observed and for which Jacobi first introduced this term, and in view of its extensive usage in the biochemical field, the authors would define "autolysis" as "the breaking down and solution of some of the essential chemical constituents of the cell by agencies (enzymes) originating within the cell." As bacteria are composed mainly of protoplasm it may be assumed that in ordinary autolysis the hydrolysis is chiefly of a proteolytic nature. It is for this reason that the present investigation has been concerned wholly with the changes which the proteins and related complex nitrogenous substances undergo.

The logical basis for any serious consideration of the phenomenon of autolysis must be a study of the chemical changes involved. The hydrolysis of protein or protein-like substances consists in the decomposition of the more complex molecules into simpler components. This constitutes a change in the

absolute and the relative amounts of the various forms of nitrogen. The present investigation is essentially an attempt to follow these changes by simple and practical chemical and physico-chemical methods.

#### METHODS AND EXPERIMENTAL PROCEDURE

The quantitative biuret test as employed by Vernon (1903) was found to be a valuable method of determining the relative amounts of protein and other closely related nitrogenous substances. Increases in amino and ammonia nitrogen were followed by the Sørensen titrations (1908). Amino nitrogen was determined also by the Van Slyke method (1911 and 1912). Observations were also made of the changes in conductivity of the autolyzing suspensions of bacteria, a procedure used by Sjöquist (1895) and later by Bayliss (1904) in their studies of digestion.

The organisms which were selected for the investigation may be placed in three distinct classes. The first is made up of those which are powerfully proteolytic, *B. subtilis*, *Erythrobacillus prodigiosus*, and related species. These are all gelatin-liquefiers. The second class is non-proteolytic and includes the colony-typhoid group. The third includes certain of the pathogenic cocci which possess little or no proteolytic power, namely *Staphylococcus aureus*, *Streptococcus pyogenes*, the pneumococcus, gonococcus and meningococcus.

The non-pathogenic organisms were grown on plain two per cent agar. Some of the pathogenic cocci required special media. A luxuriant growth of the pneumococcus, gonococcus and meningococcus was obtained by the addition of one part of ascitic fluid to two parts of three per cent agar. An even more satisfactory medium was the testicular extract agar which Hall (1916) recommends for the cultivation of the gonococcus. This medium has the advantage of not being affected by ordinary sterilization methods. The meningococcus was grown on liver extract agar in the experiments which involve amino nitrogen determinations. In one instance the pneumococcus was grown in fresh extract broth and the organisms removed by centrifuging.

Kolle flasks were employed in order to supply a maximum surface for growth. The condensation fluid was removed with a pipette and the surface allowed some time to dry, before inoculation. The inoculum was taken from young test tube cultures of the same media, and spread over the surface with a bent glass rod. The flasks were incubated for varying lengths of time, usually until the maximum growth was obtained. *Bact. coli* and the different cocci were grown at 37°C. The other cultures were incubated at 24 to 30°. The harvesting was accomplished by scraping the growth from the surface with a stiff steel wire and transferring to sterile glass-stoppered bottles. Suspensions were then made by adding sterile 0.8 per cent sodium chloride solution, or in some instances distilled water, and shaking vigorously. After filtration through sterile cotton two to five per cent of toluol was added and the bottle well shaken. After the withdrawal of a small portion of the suspension for preliminary examination, the bottles were placed in the incubator. They were shaken frequently and samples withdrawn from time to time for analysis. The different analyses were made as follows:

#### *The biuret test*

Twenty cubic centimeters of 4 per cent NaOH were measured into a Nessler tube and 2 cc. of  $\frac{1}{100}$  normal  $\text{CuSO}_4$  added. A measured amount (usually 0.1 to 0.5 cc.) of the suspension was introduced with a sterile pipette. The amount was gauged by the intensity of color that developed. The most accurate readings are made if the test is so regulated as to give a comparatively faint color. Color comparisons were made with standard tubes to which definite but varying amounts of Witte's peptone had been added. The color strength was expressed in the terms of the percentage of Witte's peptone to which the unknown was equal in biuret-giving properties. It was found advantageous to use 0.1 per cent Witte peptone solution which (within the range of 0.1 to 2 cc.) in the standard tubes gave readable differences in 0.1 cc. gradations. These represent gradations of 0.1 mgm. of Witte's peptone. One cubic centimeter is a fair amount of the unknown to use for a test. One tenth

milligram of peptone in 1 cc. is a 0.01 per cent solution; 0.2 mgm. is a 0.02 per cent solution. It is possible, therefore, to read differences of 0.01 per cent. Slight cloudiness occasionally obscured the color to some extent. It could often be removed by centrifuging or allowing the turbidity to settle out.

#### *The Sørensen test*

Five cubic centimeters of the bacterial suspension were added to 30 cc. of distilled water. After neutralization, 5 cc. of neutral formaldehyde were added, and the resultant acidity titrated with N/20 sodium hydroxide, phenolphthalein being used as an indicator. The titrations were made immediately after the formaldehyde was thoroughly mixed with the test sample. As the results were to be used for comparative purposes only, the titrations were expressed in the number of cubic centimeters required to neutralize 100 cc. of the undiluted suspension. The amount of nitrogen in milligrams may be determined by multiplying this figure by 0.7.

#### *Conductivity determinations*

These were made with 10 cc. portions of the autolyzing material and in a conductivity cell having bright electrodes. Apparatus and methods conform with those in use in ordinary class room work. The electrodes were not platinized, owing to the possibility of a disturbing catalytic action being exerted by the finely divided platinum black. The zero point on the Wheatstone Bridge as determined by the minimum buzz in the telephone receiver was consequently not as distinct as when electrodes are platinized, but it was found that with a little practice duplicate readings could be made which checked within 2 mm. Even this slight variation was reduced by taking at least six readings within the middle third of the Wheatstone Bridge scale, employing different resistances. The determinations were made at 25°C.

#### *The Van Slyke method for the determination of amino nitrogen*

This method was found to yield valuable results when certain necessary precautions were observed. The possibilities

of variation could never be sufficiently reduced, however, to place much reliance on a single determination. At least two determinations were always made, and when these gave results differing from each other by more than 10 per cent the process was repeated until satisfactory checks were obtained. The difficulties encountered were in all probability due to the presence of bacterial cells and other suspended matter in the test material. It soon became apparent that the number of oscillations had to be regulated (240 per minute) and that more than five minutes was required to obtain a complete reaction. Ten minutes was finally adopted as the routine time. This was followed by an additional shaking by hand for one or two minutes while the motor was being used to shake the Hempel pipette. The additional gas liberated at this time was also analyzed and its volume of nitrogen added to the first determination. This gave a means of judging whether the reaction had been completed in the time allowed. Because of the extra time and labor required to obtain reliable results, this method of determining the amino nitrogen proved less practical than that of Sørensen. The original method of Van Slyke (1911, 1912) was adhered to as closely as possible, and the calculations of amino nitrogen made by reference to the tables.

#### EXPERIMENTAL DATA

Early in the investigation the course of autolysis was followed with the biuret and Sørensen tests. The results appeared to indicate a direct relationship between proteolytic activity and gelatin-liquefying power of bacteria, on the one hand, and autolysis on the other. Thus, *B. subtilis* suspensions underwent complete autolysis within two to three days, while *Bact. coli* showed no changes. *Ps. pyocyanea*, while slower to undergo self digestion than *B. subtilis*, gave biuret figures which approached 0. With both of these organisms the Sørensen figures rapidly increased. In the study of *Bact. coli* material no changes could be observed in the biuret or Sørensen values (see chart 1).

Following these earlier observations, the plan of study was considerably enlarged, and efforts were made to acquire as



complete evidence as possible concerning so-called "autolysis" in certain organisms selected for the purpose. As the work concerned entirely changes going on in the complex nitrogenous substances of the dead bacterial cells, the following methods were employed and found in a large measure to supplement one another and to act as checks on each other:

1. The quantitative biuret test as used and advocated by Vernon.
2. The Sørensen method for the determination of amino nitrogen.
3. The Van Slyke method for the determination of amino nitrogen.
4. The conductivity test.
5. Staining and microscopic examination of the bacterial cells.

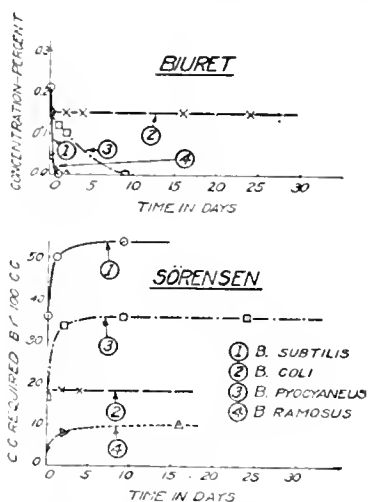


CHART I. *B. subtilis*, *B. mycoides*, *Bact. coli*, AND *Ps. pyocyanea*

The biuret readings are expressed in percentage of Witte's peptone which will give a color of equal intensity.

The Sørensen figures are expressed in the number of cubic centimeters of  $\frac{N}{30}$  NaOH required to neutralize the amino acids in 100 cc. of the solution.

### *Erythrobacillus prodigiosus*

This organism was chosen because it readily undergoes autolysis and can be easily obtained in large quantities. The accompanying charts (charts 2 and 3) show the course of changes

observed in five different experiments by the conductivity, biuret and Van Slyke methods. Number 4 was a physiological saline solution suspension of the agar surface growths, while the others were made up in distilled water. Two strains of *E. prodigiosus* were used in these tests.

The rate of autolysis was most rapid within the first twenty-four hours, but required from ten to twenty days to be practically completed. The biuret figures continued slightly

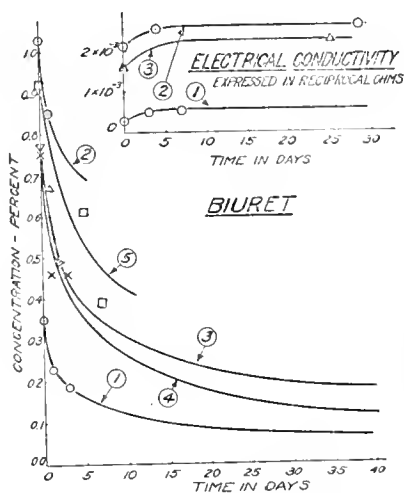


CHART 2. *Erythrobacillus prodigiosus*

Electrical conductivity is expressed in reciprocal ohms.

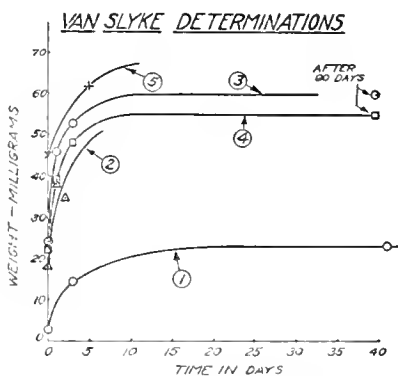


CHART 3. *Erythrobacillus prodigiosus*

The Van Slyke determinations are recorded in the number of milligrams of primary amino acids in 100 cc. of the bacterial suspension.

in the downward course even after the thirtieth day, whereas the Van Slyke values attained their maximum by the tenth day in two experiments, and by the twentieth day in a third. The other two experiments were interrupted early. The biuret and Van Slyke figures are in fairly close agreement; and both are in a large measure supported by the results of the conductivity tests. The conductivity rose during the first seven to

eight days, but remained stationary after this period. Its increase was not proportional to the amino nitrogen increase.

*Bacterium coli*

In the first study 14 experiments were conducted with six different strains of *Bact. coli*. No decrease in the intensity of the biuret reaction could be observed except in three instances in which only a slight change was apparent. In four of the tests only a small increase in the Sørensen figures was shown.

The biuret tests in this series were less delicate than those which followed. In the preparation of standards 0.25 per cent

TABLE 1

*Showing amino nitrogen changes during incubation of suspensions of Bact. coli.*

NUMBER OF EXPERIMENT	AGE OF CULTURE	ORIGINAL AMINO NITROGEN CONTENT	DURATION OF INCUBATION	FINAL AMINO NITROGEN
	<i>hours</i>	<i>mgms. per cc.</i>		<i>mgm. per cc.</i>
1	36	0.19	3 days	0.38
2	14	0.10	4 days	0.11
3	24	0.13	3 days	0.12
	24	0.13	3 months	0.11
4	48	0.08	8 hours	0.165
5	48	0.08	15 days	0.19
6	48	0.31	1 day	0.85
7	16	0.17	4 months	0.12
8	12	0.038	3 months	0.078
9	48	0.061	3 months	0.105

solutions of Witte's peptone were used, these giving a series of standards with 0.25 mgm. intervals between successive tubes. Each interval represents 0.025 on the ordinate of the biuret graph. Closer readings were not attempted.

Table 1 gives the results of Van Slyke determinations of amino nitrogen on 9 different suspensions. While they are not decisive, they do indicate that an increase in amino nitrogen may take place. In the three instances where no increase was shown the bacterial suspensions were prepared from cultures only twenty-four hours old or less, and the appropriate enzymes may not have had time to develop. The amino nitrogen content

was so small in these suspensions that the total gas volume seldom reached 1 cc., and hence considerable error may have been introduced into the determination, although all of the tests were run in duplicate.

In the following experiments on *Bact. coli* suspensions the biuret method was somewhat modified so as to make it more delicate. Instead of the 0.25 per cent basal solutions of Witte's peptone, 0.1 per cent strengths were employed, which furnished standards with 0.1 mgm. intervals, each interval being represented on the ordinate by 0.01.

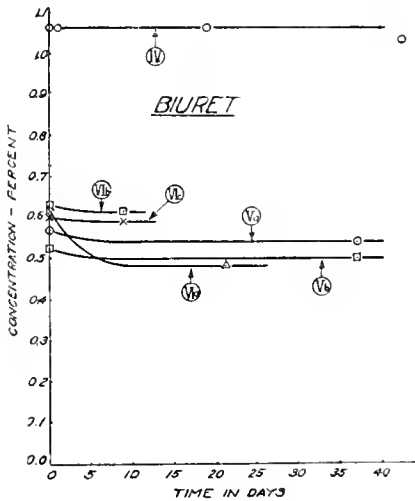


CHART 4. *Bact. coli*

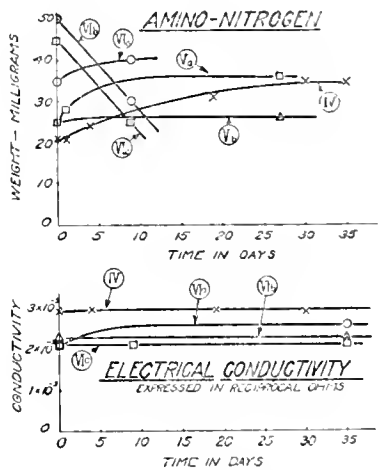


CHART 5. *Bact. coli*

Charts 4 and 5, barring curve Vla, show only a slight decrease in biuret and a corresponding increase in amino nitrogen. The biuret change in Vla was far greater than in any of the others, and is suggestive of action by enzymes other than those of *Bact. coli*, that is the presence of contaminating bacteria. It was impossible to demonstrate any contamination, though it may have existed, owing to the toluol which was added as an antiseptic, and to the inconclusive results of microscopic study.

Curves Vlb and Vlc represent control suspensions which were heated to 60° and 75°, respectively, for thirty minutes, before

incubation. Both show a slight decrease in biuret, and a very abrupt and pronounced decrease in amino nitrogen. This sharp drop in amino nitrogen is an anomaly, and no explanation is offered.

The conductivity of the suspensions of *Bact. coli* remained remarkably constant, except that of VIa which corresponded to the decrease in biuret. Of the three constant suspensions, however, two were heated controls.

The hydrogen ion concentrations of the different bacterial suspensions ranged from pH 7 to pH 9. Vb was brought to pH 9 by the addition of sodium carbonate, while Va remained of its own accord at about pH 8. Number VI had a hydrogen ion concentration of pH 7.

The results recorded here indicate that *Bact. coli* undergoes but slight autolysis, at the best, under conditions which are most favorable for this sort of enzymatic action.

#### *Staphylococcus aureus and Streptococcus pyogenes*

These two organisms are discussed together because they showed practically the same autolytic changes, and because the curves are given on the same chart (6). The results are of considerable interest in that the staphylococcus, which is an active gelatin liquefier, underwent but partial self digestion, and the streptococcus, which possessed no gelatin-liquefying property, whatever, elaborated enzymes which not only reduced the biuret appreciably, but brought about a marked increase in the amino nitrogen.

Four experiments were carried out with the staphylococcus, two different strains being employed. Curve 4 represents one strain, and 1, 2, and 3 the other. Curves I and II represent two experiments on one strain of streptococcus. All of these strains showed autolytic changes, although one of the strains of *Staphylococcus aureus* failed to give any evidence of this in one of the experiments (1). The suspensions were made in distilled water.

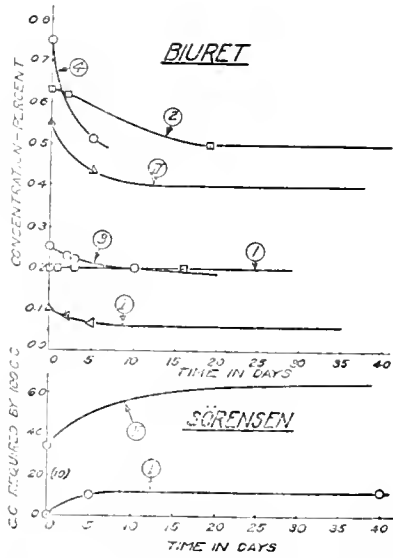


CHART 6. STAPHYLOCOCCUS AND STREPTOCOCCUS

*The gonococcus*

The following pages are given to a discussion of the changes taking place in certain of the pathogenic cocci, namely, the gonococcus, meningococcus and pneumococcus. Inspection of the

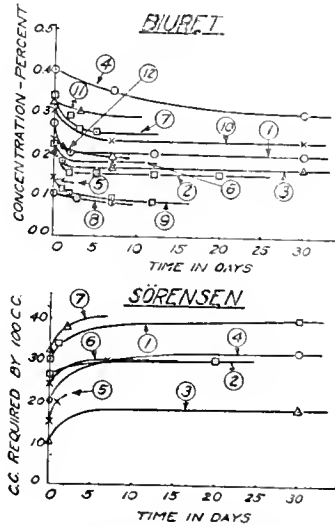


CHART 7. GONOCOCCUS

accompanying charts should leave little doubt that the process observed in these organisms is, to a certain extent at least, a true autolysis with an actual breaking down of the very complex nitrogenous substances to the amino acid stage.

Chart 7 gives the results of 12 different experiments conducted on 6 different strains of the gonococcus (2 experiments with each). Testicular extract agar and ascitic fluid agar were used, both with and without glucose, for growing the organisms. Suspensions were made in distilled water and in physiological saline solution. The curves for the biuret and Sørensen values resemble each other so closely that they need not be considered individually. It should be noted that the changes which occur develop quite rapidly. These changes in biuret and amino nitrogen were in a large measure correlated with morphological changes which could be readily observed in stained preparations of the bacterial suspensions.

#### *The meningococcus*

The meningococcus presents a picture not unlike that of the gonococcus, though there seems here to be considerably more variation in the results obtained with the different strains (see charts 8 and 9). It is of particular interest to compare the experiments in which the same strains were used, and note the near proximation of the results. Thus, 1 and 14 were the same except that 14 was kept in the refrigerator. The same strain was also used in experiments 2 and 3. Experiments 7, 8 and 12 were conducted with the same organism, and the curves both for the biuret and Sørensen values tend to be parallel. The biuret curve of 12, if shown, would coincide with that of 1. Numbers 10 and 11 are from the same strain. The biuret curve for 10, if given, would almost coincide with number 2. The organisms used in experiments 5 and 6 were obtained from the Rockefeller Institute for Medical Research, one being labeled "para," and the other "meningococcus." The curves parallel each other almost perfectly.

These experiments were conducted over a considerable period of time and often with different, media hence the strains employed constituted about the only constant factor. From a comparison of curves 7 and 8, it appears that, within certain limits at least, variations in initial concentration do not materially affect the course of the reaction.

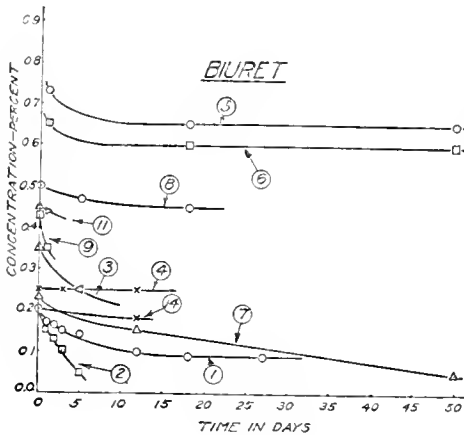


CHART 8. MENINGOCOCCUS

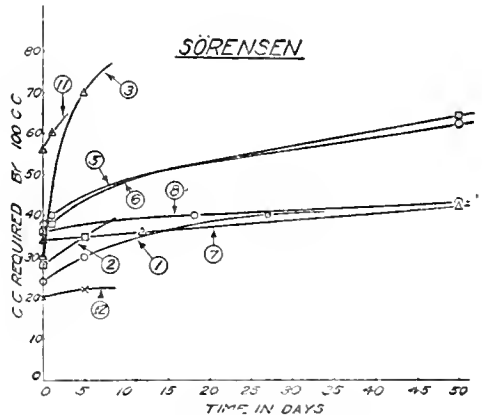


CHART 9. MENINGOCOCCUS

### *The pneumococcus*

Thirteen experiments were conducted with 4 different strains of pneumococcus. Here again autolytic changes were evident to some extent in every instance but one (see chart 10). Number 6 was a control suspension which was heated at 70°C. to destroy the enzymes. For the sake of clearness the biuret curves for 1 and 7 were omitted from the chart. Number 1 would follow number 2 so closely as to coincide with it in the first part of its course. Experiment 7 is the one in which no changes were observed. If plotted, all of the points of this curve would be found to lie on the 0.2 ordinate. The material for this particular experiment was obtained from a sugar medium and the suspension was quite acid. It was found in certain experiments not recorded here that when emulsions of the cocci prepared from glucose agar cultures showed an acidity as high as



+1.5 to +2.5 (Fuller's scale), no autolytic changes could be observed. The other curves on these graphs present a general similarity, except Sørensen 3 and 7, in spite of the fact that they represent material grown on two different media, and for varying lengths of time, and that some of the suspensions were made in distilled water and others in physiological saline solution.

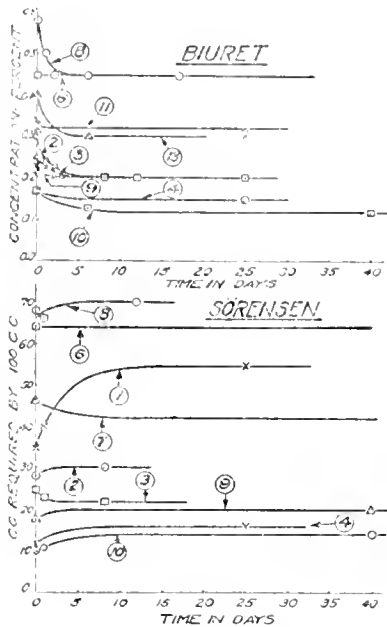


CHART 10. PNEUMOCOCCUS

*Additional experiments with the pneumococcus and meningococcus*

The following experiments were made possible by the generous coöperation of the Lederle Antitoxin Laboratories at Pearl River, New York. It was only by having use of the facilities of this laboratory and special assistance of a technical nature that the amounts of bacterial growth necessary for carrying out this work could be obtained.

The pneumococcus was grown in fresh extract broth, containing 0.1 per cent glucose, which had long been used for the

routine production of heavy growths of this organism. Thirty liters of this medium distributed in 3-liter bottles were inoculated from young broth cultures with the aid of a pipette, and incubated at 37°C. for fifteen hours, by which time a fairly luxuriant (about nephelometer 3) growth had developed. The broth culture was then run through a Sharpless laboratory centrifuge at a speed of 30,000 revolutions per minute. This separated out the bacterial cells in the form of a viscid paste, which was

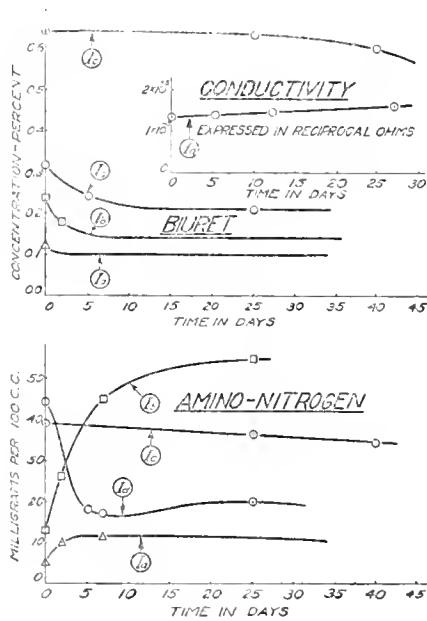


CHART 11. PNEUMOCOCCUS

scraped into a sterile, wide-mouthed, glass-stoppered bottle. About 17 grams of material were obtained which had a moisture content of 80 to 90 per cent. The bottle was packed in a freezing mixture and the contents kept near the freezing point for three days, until the experiments could be started. The bacterial paste was then divided into four parts and treated as follows:

- 1a. 0.55 grams suspended in 25 cc. physiological saline
- 1b. 1.0 grams suspended in 25 cc. physiological saline
- 1c. 5.0 grams suspended in 40 cc. physiological saline
- 1d. 2.4 grams suspended in 96 cc. distilled water

The customary aseptic precautions were taken and toluol added to the suspensions. After a vigorous shaking the bottles were incubated at 37°C., with the exception of Ic which was placed in the ice box, and the autolysis allowed to proceed. The results are shown in chart 11.

A glance at the chart shows that the 3 incubated samples underwent hydrolysis to the extent of from 20 to 40 per cent of the biuret-giving substances of the bacterial cells. The amino nitrogen determinations, however, presented peculiar anomalies. In Ia and Ib there were sufficient increases to account for the hydrolysis, but Ic and Id actually showed a decrease, the discrepancy in Id being very considerable. One-third of the entire biuret-giving material disappeared without yielding any increase in amino nitrogen as estimated by these tests. Furthermore the amino nitrogen present in the beginning seemed to decrease by about half. The ratio is nearly the same for Ic, which was kept at ice box temperature, where one-sixteenth of the biuret-giving substances were lost, with an accompanying decrease in amino nitrogen of about one-eighth. No satisfactory explanation can be offered for these apparent discrepancies. That these curves represent, in the main, the actual course of the reaction is supported by the general agreement of the many readings, not all of which have been plotted on this chart. It would seem that the first reading on the amino nitrogen curve for Id is plotted a little too high. This point was obtained from the average of two out of three determinations which gave gas volumes of 1.60, 1.74 and 1.80 cc. of nitrogen, respectively.

Conductivity determinations were also made from time to time of samples taken from the test suspensions. The results are expressed in the insert in chart 11. A slight but uniform increase in conductivity was noted throughout the experiment.

To explain the anomalous behaviour of suspensions Ic and Id the hypothesis was advanced that we are concerned here with some vigorous deamidizing action whereby the amino acids lost their identity as fast as they were formed. It was too late, however, to study changes in ammonia content, but no evidence could be adduced that there had been any considerable forma-

tion of ammonia. Analysis by the Folin micro-method revealed less than 2 mgm. of ammonia nitrogen per 100 cc. of the suspension, while there were 20 mgm. of amino nitrogen to be accounted for. The deamidizing action of the autolysate was tested on a weak solution of glycocoll, but little, if any, increase in ammonia nitrogen could be detected. The long incubation of the suspension may, however, have brought about a loss of deamidizing action by this time. The hydrogen ion concentration was, at the end of the incubation period, approximately pH 7.0, which does not suggest the liberation of any considerable amount of ammonia.

It may be of some interest to note (chart 11) the close agreement of the initial determinations of the three saline suspensions. In preparing these it was attempted to give Ib a concentration twice as strong, and Ic 6 times as strong as that of Ia. While this involved careful portioning of the pasty bacterial material, which must have varied more or less in concentration in its different parts, the first determinations of both biuret and amino nitrogen show that the 3 suspensions bore the concentration relationship of 1, 2 and 6.

A liver extract agar was employed for the massive growth of meningococcus required in the following meningococcus experiment. A very luxuriant growth developed on this medium (employed in 1-liter Blake bottles) which was removed by scraping across the surface with a heavy wire bent into a loop at the end. Thirty of the Blake bottles yielded enough growth to make 11 grams of moist scrapings. This material was transferred to a sterile glass-stoppered bottle and kept in a freezing mixture for three days, or until the experiments could be started. Five grams of the viscid material were suspended in 100 cc. of sterile distilled water (Ie). The remainder was divided and one part made into a dilute (1 gram in 30 cc.) and the other into a concentrated suspension in (2 grams in 30 cc.) physiological saline solution. For the purpose of observing the effects of incubation at 37° versus low temperature, each of these two suspensions was divided into 2 parts. Five different suspensions were used, therefore, namely;

Ia, dilute (1 gram in 30 cc. of saline), at 37°C.

Ib, dilute (1 gram in 30 cc. of saline), at ice box temperature

Ic, concentrated (3 grams in 30 cc. saline), at 37°C.

Id, concentrated (3 grams in 30 cc. saline), at ice box temperature

Ie, 5 grams of the concentrated bacterial material suspended in 100 cc. of distilled water, and incubated at 37°.

The results are shown in charts 12 and 13. The biuret curves indicate an extensive autolysis of two of the incubated samples,

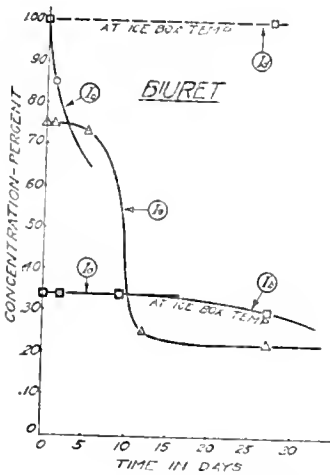


CHART 12. MENINGOCOCCUS

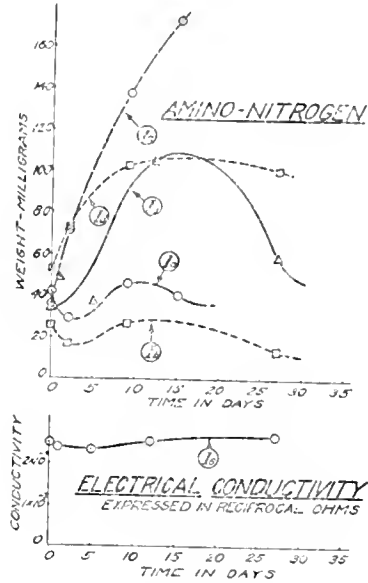


CHART 13. MENINGOCOCCUS

Ic and Ie, and but little or no change in the others. In the distilled water sample (Ie) the reduction in biuret was very slight during the first five days but subsequently progressed rapidly so that at the end of twelve days the biuret-giving substances were reduced to one-third of the original.

Owing to lack of sufficient material, duplicate amino acid determinations were made of Ie only (chart 13). All of the amino nitrogen curves have a few points of similarity, and all attain their maximum level within ten to fifteen days. Beyond this the values fall off somewhat, barring Ic of course, and, as

was observed in the pneumococcus experiments (chart 11), there appears to be some evidence of deamidization. No proof of such a process could be established, however, by further study. There is the possibility, of course, that such a deamidizing agent may have been lost from the suspension during the long and continued incubation.

Amino nitrogen curve Ie shows an apparent discrepancy. As compared with Ia and Ib, and with the biuret curve for this same suspension, the two and the five day points should be practically on the same level.

The conductivity readings for suspensions Ie show very slight changes.

#### GENERAL DISCUSSION

The rapid and profound autolysis of the liquefying bacteria, particularly *Erythrobacillus prodigiosus*, which could be readily demonstrated by the methods employed in this investigation, is to be expected, but that such well-known non-liquefying organisms as *Bact. coli*, the gonococcus, meningococcus and pneumococcus, should undergo a similar process, though in a much lesser degree, evoked considerable surprise in the authors. In order to satisfy themselves as to the ability of the pathogenic cocci to attack gelatin under the most favorable conditions of environment and temperature, several fruitless attempts were made to induce gelatin liquefaction in enriched media.

The enzymes involved in the autolysis of the above-mentioned non-liquefying organisms cannot but have a specific or at least a limited sphere of action. According to Rosenow (1912) the pneumococcus is able to attack the proteins of ascitic fluid, blood serum and meat extract, but we have been unable to demonstrate any proteolysis of albumin, casein or gelatin by the pneumococcus, with the use of the more delicate and specific methods employed in this work.

It has been assumed throughout the investigation that enzymes, when present, would assert themselves in a neutral or weakly alkaline medium. These experiments have as a rule been conducted, therefore, in hydrogen ion concentrations rang-

ing from pH 7.0 to 9.0. A series of tests made upon *E. prodigiosus* in different hydrogen ion concentrations showed that, although slow digestion took place at pH 4.0, the most rapid digestion occurred at pH 7.0. Rosenow claimed that the enzyme action demonstrated by him in the pneumococcus is favored in a +0.5 medium (phenolphthalein titration). Dernby (1917 and 1918), held, on the other hand, that a pH of 6.0 was the most favorable for the autolysis of animal organs.

The very slight changes which were observed in the *Bact. coli* suspensions are of particular interest and solicit further inquiry. In the first set of experiments very little enzymatic action of any sort could be demonstrated, and then in 4 of the suspensions only. In the second series (Charts 4 and 5) a slight reduction in biuret (1 per cent to 6 per cent) and an increase in amino acids were shown in every instance barring experiment VIa from which the possibility of contamination cannot be excluded. The chemical changes in these suspensions were so slight as not to be indicated at all by the conductivity tests. As the 2 heated controls showed almost the same change as the unheated test samples the "autolysis" manifestations become all the more insignificant. It is possible, however, that the heating at 60° and 75°C. may not have been sufficient to destroy the enzymes. Abbott and Gildersleeve (1903) found that heating at 100° for fifteen to thirty minutes did not always destroy certain bacterial enzymes. Meyer (1911) reported that the proteases of *Pseudomonas pyocyanea* are little affected by 15 minutes of exposure to 100°C. Wells and Corper (1912) describe lipolytic enzymes in *Mycobacterium tuberculosis* which are not entirely destroyed in thirty minutes at 100°. On the other hand, Fermi (1894) found 55 to 70° to be sufficient to destroy the proteolytic enzymes of bacteria.

The possibility of slight decreases in biuret and increases in amino nitrogen from causes other than autolysis must not be ignored. If, in the process of harvesting the bacterial growths any appreciable amount of peptone with its various biuret-giving substances should be removed from the agar medium, and if the suspended organisms elaborate enzymes which act on any

of these biuret-containing substances the results of such action would be revealed in subsequent tests. In fact, it is more than possible that the bacterial cells contain relatively simple biuret-giving polypeptides which have been absorbed from the medium or which are intermediate synthetic products occurring in the natural course of bacterial metabolism. Berman and Rettger (1918) showed that *Bact. coli* is unable to utilize the more complex or proteose fraction of Witte's peptone, but that some of the simpler biuret-containing substances in the commercial peptone, probably polypeptides, are attacked by pure culture of this organism and broken down into the simpler amino acids.

Furthermore, it is doubtful whether very slight increases in amino nitrogen need necessarily indicate any hydrolysis. We may be dealing with more or less masking of amino nitrogen by the protein, which does not allow all of the amino nitrogen to react in the Van Slyke procedure, during the early part of the incubation period. Such a protective action may to a certain extent be removed by such purely physical processes as diffusion, partial disintegration from osmotic disturbance, toluol extraction, etc. Changes of this type would render the amino acids more and more available during the course of incubation for the Van Slyke determination. Such influences scarcely seem sufficient, however, to explain the more pronounced increases in amino nitrogen noted in some of the experiments, as for example in chart 6 where the increases are shown to range from 4 to 60 per cent.

The failure to demonstrate marked changes in the suspensions of *Bact. coli* by the biuret, Sørensen, Van Slyke and conductivity methods is fully supported by direct microscopic examinations of the different suspension during the periods of observation. The individual cells at all times took the ordinary stains well and with a high degree of uniformity, and there was no evidence of morphological change, except in VIa of the second series of experiments which also differed markedly from all others in its reaction to the biuret, Van Slyke and conductivity tests, and which must be excluded from serious consideration on account of the probability of bacterial contamination.



As so few tests were made with the *Staphylococcus* and *Streptococcus* no further comment should be necessary than to state in review that both of these organisms showed distinct evidence of elaborating enzymes which have appreciable autolytic action, especially the *staphylococcus*.

The results obtained with the pneumococcus, gonococcus and meningococcus indicate that these organisms undergo autolytic changes as the result of their own enzyme action, and particularly the meningococcus and the gonococcus. This is further substantiated in the case of the two last mentioned organisms by the microscopic examination of stained slides in which decreased staining ability and change in cell morphology are shown during the course of the autolysis, as had been observed previously by Flexner (1907) for the meningococcus.

#### SUMMARY AND CONCLUSIONS

It has been possible to follow in a quantitative way the nitrogenous changes taking place in autolyzing bacterial suspensions. In this study the quantitative biuret test of Vernon and the Sørensen titration have proven of the greatest value. The Van Slyke determination of amino nitrogen has also been employed to advantage, although the material to be analyzed was not particularly suited to this method. Electrical conductivity has been found to increase during autolysis, but its increase has not been proportional to the amount of amino nitrogen formed by the hydrolysis of protein, as Bayliss (1907-8) claims to have found to be the case in the tryptic digestion of caseinogen and gelatin.

By the application of such methods it has been possible to show that:

1. Proteolytic bacteria of the type of *Erythrobacillus prodigiosus*, *Pseudomonas pyocyanea*, and *B. subtilis* autolyze rapidly.
2. *Bact. coli* undergoes slight changes which may be autolytic in nature, but which at best involve only a small part of the complex nitrogenous constituent or constituents of the cells.
3. The pathogenic cocci, pneumococcus, gonococcus and meningococcus undergo an actual autolysis with a breaking down of the protein or protein-like substances of the bacterial cells.

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BACILLUS HEMOGLOBINOPHILUS CANIS  
(FRIEDBERGER)

(*HEMOPHILUS CANIS EMEND.*)

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Friedberger in (1903), working in Pfeiffer's clinic reported finding in the preputial secretions of a dog a small, Gram-negative, non-motile, hemoglobinophilic bacillus which he called *B. hemoglobinophilus canis*. From his description it is difficult to see how he differentiated it from *H. influenzae* except that he isolated it from a dog instead of a human being. Odaira (1912), also working with Pfeiffer, compared *H. pertussis*, *H. influenzae* and *H. canis*, and found that by agglutination tests they were different. When complement fixation tests were used less specificity was shown.

Since the last pandemic of influenza much interest has been taken in the so-called hemophilic bacilli in regard to their relation to disease, their growth requirements and their biological reactions. It seemed, as advance had been made in differentiating many of these organisms by biological reactions, that *H. canis* might also be differentiated culturally from closely allied organisms. During the past year six strains have been isolated from the preputial secretions of dogs. Most of the male dogs examined had a certain amount of pus in the preputial secretions. Direct smears of this pus often showed numerous small Gram-negative bacilli and when cultured *H. canis* was usually found to be the predominating organism. Up to the present this organism has been isolated from dogs only, but it is conceivable that laboratory workers and people who handle dogs might show this bacillus accidentally in throat or nose cultures.

*Morphology, staining, motility.* It is a small pleomorphic, Gram-negative, non-motile rod which looks very much like *H. influenzae*.

*Type of growth.* On 2 per cent rabbit-blood meat-infusion agar young colonies are non-hemolytic, round, with a small granular area on top. At this time they are indistinguishable from colonies of *H. influenzae* but as they grow older the former become distinctly more opaque than the latter. Old cultures on blood agar slants show a luxuriant opaque growth that resembles *H. pertussis* more than *H. influenzae*. A diffuse growth occurs in the proper liquid medium.

*Growth requirements.* *H. canis* grows well on 5 per cent human blood agar. This is a distinct difference between *H. canis* and *H. influenzae* as the latter does not grow well on media containing fresh unheated human blood (Rivers, 1919). *H. influenzae* (Rivers and Poole, 1921) requires two food accessory factors, one autoclave labile, the other autoclave stable. *H. canis*, however, requires the addition of only the autoclave stable substance as an accessory factor. It will not live more than one or two generations on meat infusion agar, meat infusion agar plus ascitic fluid or 2 per cent peptone agar plus yeast extract. Successful transplants can be made indefinitely on meat infusion agar plus hematin or 2 per cent peptone agar plus hematin. It is aerobic.

*Indole production.* All the strains produced indole.

*Nitrate reduction.* All the strains reduced nitrates to nitrites.

*Reaction in milk.* Very little change was noticed in blood milk mixtures when inoculated with *H. canis*.

*Sugar fermentations.* The medium used for fermentation tests was the same as described in a previous paper. (Rivers and Kohn, 1921). All the strains formed acid without gas from glucose, fructose, galactose, mannitol, sucrose, and xylose. Neither acid nor gas was formed from maltose, lactose, dextrin, arabinose and glycerol.

*Hemolysis.* Red blood cells were not hemolyzed in solid or in liquid media by any of the strains.

*Pathogenicity.* One cubic centimeter of a twenty-four-hour culture in blood broth did not kill a white mouse when given intraperitoneally; two cubic centimeters given intraperitoneally did not kill a small guinea pig; one cubic centimeter intravenously did not kill a small rabbit.

From table 1 it can be seen that *H. canis* can be differentiated from *H. pertussis* by indole production, nitrate reduction and sugar fermentations; from *H. influenzae* by growth accessory factors and mannitol fermentation.

TABLE 1

*Most important differential cultural characteristics of H. pertussis, H. influenzae and H. canis*

BACTERIUM	ACCESSORY GROWTH FACTORS	INDOLE PRODUCTION	NITRATE REDUCTION	SUGAR FERMENTATIONS
<i>H. pertussis</i>	Old cultures require neither autoclave labile nor autoclave stable factor	Never produced	Never reduced	No sugar fermented
<i>H. influenzae</i>	Autoclave labile and autoclave stable factors required	May or may not be produced	Always reduced	Different sugars fermented; never mannitol
<i>H. canis</i>	Only autoclave stable factor required	Always produced	Always reduced	Different sugars fermented, including mannitol

## CONCLUSIONS

*B. hemoglobinophilus canis* of Friedberger has been further described and differentiated from *H. pertussis* and *H. influenzae* by cultural methods. According to the new classification this bacillus should be called *Hemophilus canis*.

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## SALT EFFECTS IN BACTERIAL GROWTH

### III. SALT EFFECTS IN RELATION TO THE LAG PERIOD AND VELOCITY OF GROWTH<sup>1</sup>

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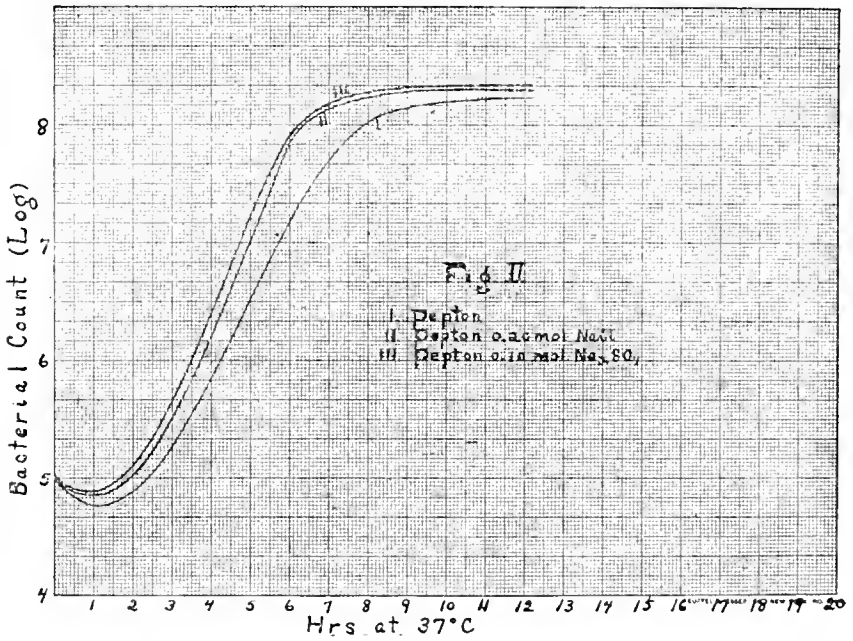
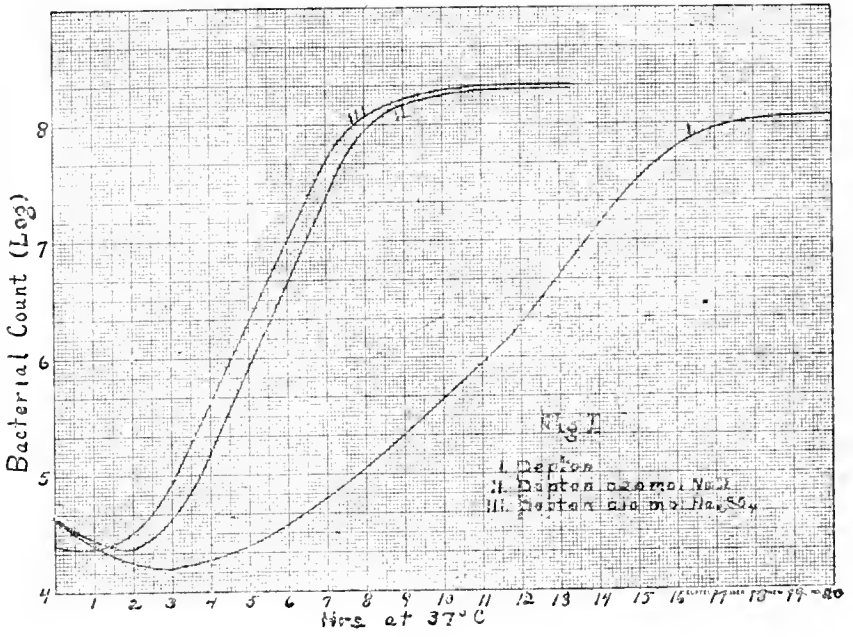
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In a previous paper of this series (Holm and Sherman, 1921), it has been shown that certain neutral salts, in proper concentrations, accelerate the growth of *Bact. coli*, as measured by the time required to produce visible turbidity, the time required to reduce methylene blue, and the rate of acid production in the presence of a fermentable carbohydrate.

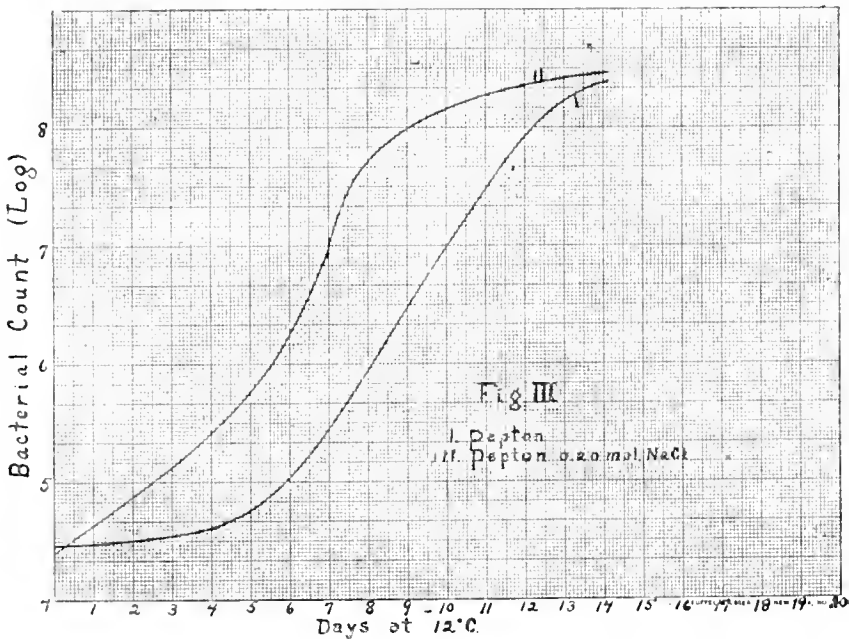
In the present work we have extended these experiments by the use of the plate count in an effort to throw more light on the mechanism of the salt action. Since our previous experiments have shown that the salt effect is magnified on the acid side of the region of optimum growth (Sherman and Holm, 1922), we have used in the present work media adjusted to a reaction of pH 5.4. All of the counts here reported represent the average of triplicate plates on extract-pepton agar incubated for three days at 33°C. Further details of the experiments are given in the appendix.

From figures 1 and 2, plotted from the data obtained in experiments 1 and 2 in which the growth of *Bact. coli* in 1 per cent pepton, 1 per cent pepton plus 0.2 M NaCl, and 1 per cent pepton plus 0.1 M Na<sub>2</sub>SO<sub>4</sub> was measured, it is seen that the accelerating action of the salts is due to an increase in the velocity of growth of the organisms. In other words, the period of logarithmic growth is shortened since the number of bacteria present in the

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culture at the breaking point of the growth curve is approximately the same in all cases. It also appears that the salts have the effect of shortening the latent period previous to rapid growth. This difference is marked in the case of the sulphate (0.1 M), which appears to have a somewhat greater accelerating effect than the chloride, while in the case of the NaCl (0.2 M) the shortening effect upon the period of lag is not so definite; or may



be entirely lacking, as is indicated by the results of experiment 2.

The effect of NaCl upon the period of lag was therefore extended in experiments 3 to 6 in which plate counts were made at hourly intervals. The results of these additional experiments again show that while NaCl may or may not decrease the latent period, it increases in every case the velocity of growth during the period of active multiplication.

It was thought that it might be possible to magnify the effect of NaCl by incubation at a temperature which allowed only a

slow multiplication of the organisms. This was done in tests in which cultures were incubated at a temperature of 12°C. At this temperature the increments of growth when measured daily are about the same as those taken at hourly intervals at 37°C. The results obtained in these tests (experiments 7 and 8) show the same characteristic increase in the velocity of growth with NaCl, and also a well defined shortening of the lag period. The data from experiment 7 are plotted in figure 3.

#### SUMMARY

It has been shown that the accelerating effect of certain salts upon the growth of *Bact. coli* is due primarily to an increase in the velocity of growth of the organism during the period of maximum multiplication.

The same salts usually also increase the accelerating effect by decreasing the duration of the preliminary latent period.

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#### APPENDIX

The organism used in all of these experiments was a laboratory strain of *Bact. coli*. Inoculations were made from cultures one or more days old in 1 per cent pepton.

The media used for determining the growth rates were put up in 100-cc. amounts; all contained 1 per cent pepton, with the indicated amount of salt, and were adjusted to a reaction of pH 5.4.

In experiments 1 to 6, incubations were at 37°C. and plate counts were made at hourly intervals. Experiments 7 and 8 were conducted at 12°C. and counts made at daily intervals.

Standard extract-pepton agar was used for plating, and the plates were incubated for three days at 33°C. before counting. Triplicate plates of each dilution were made in every case.

*Experiment 1*

HOURS	NUMBER OF BACTERIA PER CUBIC CENTIMETER		
	Pepton	0.20 M NaCl	0.10 M Na <sub>2</sub> SO <sub>4</sub> M Na <sub>2</sub> SO <sub>4</sub>
0	39,000	41,000	26,000
1	29,000	28,000	21,000
2	17,000	23,300	33,700
3	16,000	48,000	86,000
4	17,000	163,000	473,000
5	33,000	620,000	2,630,000
6	47,000	4,700,000	12,300,000
7	66,000	29,300,000	76,000,000
8	117,000	135,000,000	153,000,000
9	210,000	164,000,000	149,000,000
10	410,000		183,000,000
11	780,000	188,000,000	198,000,000
12	1,560,000	208,000,000	183,000,000
13	4,300,000	231,000,000	220,000,000
14	12,100,000		
16	67,000,000		
18	104,000,000		
20	121,000,000		

*Experiment 2*

HOURS	NUMBER OF BACTERIA PER CUBIC CENTIMETER		
	Pepton	0.20 M NaCl	0.10 M Na <sub>2</sub> SO <sub>4</sub> M Na <sub>2</sub> SO <sub>4</sub>
0	99,000	95,000	103,000
1	58,000	79,000	78,000
2	77,000	106,000	125,000
3	300,000	181,000	471,000
4	766,000	1,750,000	2,630,000
5	3,730,000	8,500,000	16,300,000
6	17,900,000	74,000,000	74,000,000
7	49,500,000	172,000,000	168,000,000
8	109,000,000	181,000,000	191,000,000
9	149,000,000	169,000,000	201,000,000
10	148,000,000	190,000,000	209,000,000
11	162,000,000	207,000,000	214,000,000
12	175,000,000	214,000,000	215,000,000

*Experiment 3*

HOURS	NUMBER OF BACTERIA PER CUBIC CENTIMETER	
	Pepton	0.20 M NaCl
0	54,000	56,000
1	52,000	52,000
2	49,000	76,000
3	86,000	129,000
4	152,000	480,000
5	360,000	780,000
6	790,000	14,100,000
7	3,200,000	53,000,000

*Experiment 4*

HOURS	NUMBER OF BACTERIA PER CUBIC CENTIMETER	
	Pepton	0.20 M NaCl
0	56,000	57,000
1	51,000	54,000
2	59,000	59,000
3	94,000	57,000
4	148,000	139,000
5	273,000	1,120,000
6	790,000	20,600,000
7	23,700,000	105,000,000

*Experiment 5*

HOURS	NUMBER OF BACTERIA PER CUBIC CENTIMETER	
	Pepton	0.20 M NaCl
0	35,000	44,000
1	41,000	42,000
2	87,000	83,000
3	310,000	203,000
4	1,090,000	423,000
5	8,100,000	23,800,000
6	23,500,000	64,000,000
7	29,200,000	63,000,000

*Experiment 6*

HOURS	NUMBER OF BACTERIA PER CUBIC CENTIMETER	
	Pepton	0.20 M NaCl
0	126,000	119,000
1	128,000	132,000
2	175,000	188,000
3	676,000	940,000
4	2,660,000	4,440,000
5	17,800,000	85,000,000
6	24,500,000	135,000,000
7	45,200,000	205,000,000

*Experiment 7*

DAYS	NUMBER OF BACTERIA PER CUBIC CENTIMETER	
	Pepton	0.20 M NaCl
0	28,200	25,300
1	29,000	37,500
2	38,000	69,000
3	38,600	161,000
4	47,000	240,000
5	46,000	523,000
6	129,000	1,630,000
7	360,000	14,750,000
8	834,000	54,000,000
9	3,100,000	96,000,000
10	9,300,000	150,000,000
11	45,000,000	185,000,000
12	73,000,000	148,000,000
13	156,000,000	280,000,000
14	260,000,000	290,000,000

*Experiment 8*

DAYS	NUMBER OF BACTERIA PER CUBIC CENTIMETER	
	Pepton	0.20 M NaCl
0	26,800	30,000
1	29,500	36,000
2	41,000	57,000
3	36,000	182,000
4	52,000	257,000
5	53,000	340,000
6	186,000	1,480,000
7	860,000	16,600,000
8	2,020,000	54,000,000
9	7,300,000	91,000,000
10	35,000,000	160,000,000
11	56,000,000	160,000,000
12	88,000,000	186,000,000
13	165,000,000	250,000,000
14	250,000,000	270,000,000

FURTHER OBSERVATIONS ON "COLOR STANDARDS"  
FOR THE COLORIMETRIC DETERMINATION  
OF H-ION CONCENTRATION

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The following additional observations on color standards for the colorimetric determination of H-ion concentration have been made by the author since the publication of his first article on this subject (Medalia, 1920).

It will be recalled that in the first article just referred to a method was described of preparing "color standards" for the principal indicators of Clark and Lubs. This was done by using varying amounts of indicator in acid and alkaline solutions choosing such solutions as would bring out the acid and alkaline colors of the double colored indicator. Seven pairs of tubes were used; each pair containing 0.8 cc. of indicator solution in the ratio of 1:7, 2:6, 3:5, etc., increasing by 0.1 cc. in the alkaline solution up to 0.7 cc. and decreasing by 0.1 cc. in the acid solution down to 0.1 cc. These pairs of tubes were viewed by looking through them preferably in a comparator block, when placed one behind the other, i.e., superimposing the acid and alkaline colors of the indicator in the varying strengths—similar to the superimposing method used by Sahn (1904) to determine the half transformation point, and by Barnett and Chapman (1918) to prepare the color standards for phenol red. The pH represented by the color of each pair of "color standards," was calibrated by using buffer mixtures of known H. I. C., prepared according to Clark and Lubs (1917).

The possibility suggested itself of making use, in addition to the seven pairs of "color standards" just referred to, of an

*acid* and an *alkaline* tube at either end of each set of the color standards. This would add two more colors to the range of each indicator and thus greatly enhance the usefulness of each set of color standards, more especially as regards the two *end* pairs.

Such tubes would contain each 10 cc. of the acid and alkaline solutions, respectively, as used for the particular indicator, to each of which would be added in turn 0.8 cc. of the "indicator watery solution." They would give the full acid and full alkaline colors of the particular indicator and would thus be of value in determining whether a given unknown falls within the visual or "virage" range of the particular indicator or not.

There were two questions, however, that had to be determined experimentally with reference to this:

1. Whether there would be a difference in color sufficiently marked to distinguish between such a tube, on the acid side, when compared with the first pair of the "color standards;" and the tube, on the alkaline end, when compared with the seventh pair of the set.

2. Whether any definite pH could be attributed to such tubes when studied by calibrating them with buffer mixtures of known H-ion concentration.

The first question can be answered in the affirmative: There is, according to the findings, a definite and distinct difference between the tubes on either side of the scale as compared with the respective pairs nearest to them. The solution in the tube on the acid end of the scale is the same as that described for the acid tubes of the pairs in each of the sets of "color standards" for each indicator. Similarly the tube on the alkaline end of the scale contains the solution used for the alkaline tubes of the pairs in each set.

Thus with *thymol blue* (acid range), the acid tube of this indicator is made with 0.5 volume per cent of concentrated HCl, the alkaline tube of this indicator contains 0.001 per cent concentrated HCl and to each of these is added 0.8 cc. of 0.02 per cent of the indicator watery solution.



With *brom thymol blue*: The acid tube contains 10 cc. of 0.1 per cent concentrated HCl and the alkaline tube N/200 NaOH solution.

The acid and alkaline tubes of *methyl red*, *brom cresol purple*, *brom thymol blue*, and *cresol red*, are made with 0.1 per cent concentrated HCl and N/20 NaOH, respectively. To each, of course is added 0.8 cc. of the respective indicator solution, in dilutions given in the previous article for the preparation of the color standards. The acid and alkaline tubes of *phenol red* contain 0.1 per cent HCl, and N/100 NaOH respectively,<sup>1</sup> and those of *thymol blue* (alkaline range) were made with 0.001 per cent concentrated HCl and N/20 NaOH respectively.

As to the second question: whether these tubes at the acid and alkaline ends of each set could be shown to represent a definite H-ion exponent, the tubes were matched against buffer mixtures of known H-ion concentration and showed the results indicated in the appropriate column of table 1.

With *methyl red*: The "alkaline tube" practically matched a pH 6.0 buffer mixture, the latter showing a very slightly more reddish tinge. With *phenol red*: The "acid tube" practically matched a pH 6.8 buffer mixture, approaching a pH 6.6 buffer mixture (there seems to be only a very slight difference in color between pH 6.6 and pH 6.8 with this indicator while, pH 6.4 of buffer and pH 6.6 are alike in color). With *cresol red*: The "acid tube" is very slightly clearer or brighter in color than a pH 7.2 buffer mixture, being practically the same otherwise. With *thymol blue* (alkaline range): The "acid tube" is slightly more yellowish in color than a pH 8 buffer mixture approaching pH 7.8 of the buffer. There is a marked difference in color between this tube and pair no. 1 of the set. The "alkaline tube" matched the pH 9.6 buffer mixture. There is a difference in color between this tube and pair no. 7, of the set.

The value of these "acid" and "alkaline" tubes, at the end of each set of "color standards" is not so much as to the *actual pH* which they represent, but rather in the fact that they allow a

<sup>1</sup> The N/100 NaOH was found to be the better strength to use for the alkaline tubes for the color standards of this indicator rather than N/20 NaOH as recommended in the previous article.

TABLE I  
*Data for the preparation of the "color standards", and the pH which they represent*

INDICATOR WATERY SOLUTION		TRYMOL BLUE (ACID RANGE)	BROM PHENOL BLUE	METHYL RED	BROM CRESOL PURPLE	BROM THYMOL BLUE	PHENOL RED	CRESOL RED	THYMOL BLUE (ALKALI RANGE)
Acid tube	Alkali tube								
cc.	cc.	pH							
0.8	0	1.2	3.2	4.4	5.2	6.2	6.8	7.2	8.0
0.7	0.1	1.4	3.4	4.7	5.4	6.4	7.0	7.4	8.2
0.6	0.2	1.6	3.6	4.9	5.6	6.6	7.2	7.6	8.4
0.5	0.3	1.8	3.8	5.1	5.9	6.8	7.4	7.8	8.6
0.4	0.4	2.0	4.0	5.2	6.1	7.0	7.6	8.0	8.8
0.3	0.5	2.2	4.2	5.4	6.3	7.2	7.8	8.2	9.0
0.2	0.6	2.4	4.4	5.6	6.5	7.4	8.0	8.4	9.2
0.1	0.7	2.6	4.6	5.8	6.7	7.6	8.2	8.6	9.4
0	0.8	2.8	4.8	6.0	6.8	7.8	8.4	8.8	9.6
Per cent in Indicator.*		0.02	0.02	0.02	0.02	0.02	0.04	0.02	0.02
Watery solution									
Per cent solution to pro-		0.001	N/200	N/20	N/20	N/20	N/100	N/20	N/20
duce alkaline color, in		HCl	NaOH	NaOH	NaOH	NaOH	NaOH	NaOH	NaOH
10-cc. amounts									
Per cent solution to pro-		0.5	0.1	0.1	0.1	0.1	0.1	0.1	0.001
duce acid color, in		HCl	HCl	HCl	HCl	HCl	HCl	HCl	HCl
10-cc. amounts									
Color of the "color		Red	Yellow	Red	Yellow	Yellow	Yellow	Yellow	Yellow
standards"		through	through	through	through	through	through	through	through
		red-yel-	pink-red	red-yel-	pink to	green to	pink to	pink to	green to
		low to	to blue	low to	purple	blue	red	red	blue
		yellow		yellow					blue

\* Made from alcohol 0.2 per cent stock solution, for the preparation of which see body of paper, under heading of "stock alcoholic solution."

more correct measurement of an unknown when it matches or nearly matches pair no. 1, or pair no. 7, of the "color standards."

The actual pH which the "acid" and "alkaline" tubes represent cannot be assigned to an unknown: because the unknown may be much more acid or much more alkaline than the "acid" or "alkaline" tubes at either end of the set although it does not show a different color. The value of these end tubes therefore lies in the fact that there is a distinct difference in color between the "acid tube" and the first pair, of the set on one side, and the "alkaline tube" and the last pair (pair no. 7), at the other end. The color of an unknown can be better judged when found to be between the "acid tube" and the *first pair*, or between the *last*

TABLE 2

Clark's table giving the amounts in cubic centimeters of N/20 NaOH per decigram (0.1 gram) of indicator powder

MOLECULAR WEIGHT	INDICATOR	N/20 NaOH PER DECIGRAM
		cc.
354.17	Phenol red	5.7
669.82	Brom-phenol blue	3.0
382.17	Cresol red	5.3
540.01	Brom-cresol purple	3.7
466.30	Thymol blue	4.3
624.12	Brom-thymol blue	3.2
269.12	Methyl red	7.4

*pair* and the "alkaline tube," but such judgment should be confirmed by the use of another indicator next to the one used.

It was thought best to bring together all the data necessary for the preparation of the "color standards" with the additional "end tubes" in the form of a table, which would also show the pH values which these "color standards" represent. This will facilitate the use of the colorimetric determination by means of the "color standards" described in the previous article and the additional colors described in the present article. These data may be found in table 1.

The author's attention has been called to certain minor difficulties which various workers have experienced in using the "color standards" described in the previous article already

referred to. The most important of these was the difficulty experienced in dissolving the powdered indicators as directed for the preparation of the "stock alcoholic solution." Such difficulties can easily be overcome by using N/20 NaOH in the amounts recommended by Clark (1920) which are the molecular equivalents of each indicator, for the alcoholic stock solution (see table 2).

#### "STOCK ALCOHOLIC SOLUTION"

##### *Method of preparation*

The powder (say, 0.1 gram) is weighed out on an analytical balance directly in a clean, dry, heavy-walled test tube, a few drops of the N/20 NaOH is added and the powder is rubbed up with a glass rod; the rest of the required amount of the N/20 NaOH is gradually added. The dissolved indicator is now washed into a 50-cc. graduate with 95 per cent ethyl (ordinary) alcohol, and is made up to 50 cc. with the alcohol, making a 0.2 per cent "stock alcohol solution." The "indicator watery solution" is prepared from the stock alcoholic solution as already described in the previous article.

There has recently appeared an exhaustive study on "The Reaction of Culture Media" by Bunker and Schuber (1922). These authors thoroughly discuss the question of H-ion concentration with particular reference to culture media, and in referring to the author's method, mention that their "indicator watery solution" spoiled in a few days. The "indicator watery solution" has kept well in this laboratory since October, 1919 (for nearly two and one-half years), and is still in good condition. To secure this satisfactory result the author feels that it is necessary to observe a fair amount of aseptic technic. The same sterile pipette can be used for one day for the same indicator, by keeping the pipette upright in a sterile test tube.

#### REQUIREMENTS FOR THE PREPARATION OF "COLOR STANDARDS"

The requirements for preparing a set of "color standards" for the colorimetric determination of the H-ion concentration

of any double color indicator, which would be useful and workable are:

1. The alkaline and acid solution shall be such as will bring out the alkaline and acid colors of the indicator in a well defined way.

2. The solutions used shall be of a strength that will not fade quickly.

3. They shall be of a strength that will not bring out secondary colors.

4. The indicator solutions shall be of a strength such that when made use of to prepare the color standards in the manner described they shall produce definite, strong and unmistakable color differences between the adjacent pairs at an interval of pH 0.2.

5. The amounts of indicator used in preparing the color standards shall be measured in a way that can be correctly duplicated at any time. This can best be done by using fractions of a cubic centimeter from a graduated pipette.

6. The different colors, as represented by each pair of tubes of the color standards, shall be calibrated with buffer mixtures, of known H-ion concentration containing the same amount of the buffer mixture as is present in *each* tube of the pair and exactly the same amount of indicator solution present in both tubes of the pair.

7. The work of calibrating the "standard colors" shall be done with a comparator block having 9 holes, permitting of having on each side of the "unknown" a tube with 10 cc. of buffer mixture of known H-ion concentration containing 0.8 cc. of indicator watery solution.

When measuring an unknown solution that is turbid or colored, an extra tube of this unknown should be placed in front of each standard color in the comparator block.

8. The test tubes used for the color standards must be of clear glass and of a caliber (14 to 15 mm. inside dimensions) such as to give sufficient density of color and to be as nearly alike in diameter as possible.

All these requirements the author has tried to meet and has met in the "color standards"—as previously described.

A criticism (Gillespie, 1921) of this method of preparing color standards based on theoretical mathematical calculations has no weight, if the colors, actually prepared as described, do actually match other solutions of known pH. The calibration of each "pair" in the author's series has been made by such actual comparisons, and the pH values assigned are based upon actual visual agreements with known standards and not from hypothetical relations.

As to the criticisms brought against using these pH measurements for the study of acid production by bacteria, it is the author's point of view, that the value of being able to study from hour to hour the change produced in media by a given culture without having to disturb the sterility of the culture, and the ability to measure such change in reaction in either direction in such a simple way that can be duplicated, is beyond measure. From that point of view, it is not the question of "*how much*" acid is produced, in an abstract way, in which we are interested; but rather the change in reaction, produced, biologically, in a culture medium suited for the growth of a particular organism, in a relative way. This is of particular value if it can be done without contaminating the culture or introducing foreign elements.

It is important that the experimenter state, however, what method is used in determining acid or alkali production by bacteria, and for that matter in making use of the pH measurement for any other purpose. This will avoid confusion when the work is duplicated by others.

In concluding I wish to express my appreciation to Prof. S. C. Prescott, and Prof. J. W. M. Bunker, and Dr. M. P. Horwood of the Department of Biology and Public Health of the Institute for their helpful assistance and criticism rendered me in this work.

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## THE CAUSE OF EXPLOSION IN CHOCOLATE CANDIES<sup>1</sup>

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During the past three years a number of instances of explosion of chocolate candies have been brought to the writer's attention. It appears that explosion in chocolates is rather a common occurrence, that it affects a considerable percentage of certain lots, and that few if any chocolate candy-makers escape the difficulty. If the chocolates are sold soon after they are made the trouble does not have time to develop, but concerns engaged in wholesale manufacture are likely to be caught at times. In case the trouble develops, the candy must be sold at a lowered price; or it is worked over into other products. In either case the economic loss is sufficiently great to merit a careful investigation of the problem.

### SURVEY OF LITERATURE

An extended search of the literature has been made, but no reference dealing directly with this problem has been found. A number of investigators, notably Prescott, Stiles, and Cummins, have dealt with the sanitary aspects of the candy industry, but the explosion of chocolates was not noted.

The trade journal, *Western Confectioner*, July, 1920, contains a two-page article by J. P. Booker, in which he says: "After working on the problem for fourteen years, I am convinced that the bursting of chocolates is caused by the germ coli." The conclusion is said to be based upon the work of several chemists. The germ is supposed to come from water and from starch.

<sup>1</sup> Presented at seventy-third annual meeting, Society of American Bacteriologists, Dec., 28, 1921

## DESCRIPTION OF PHENOMENON

The explosion of the chocolates occurs some time after their manufacture, usually ten days to two weeks or longer. At times the chocolate coating is merely cracked while at others it is broken into a number of fragments. If the fondant used is very moist leakage may occur. Under any circumstances the centers of the candies are exposed, the product presents an untidy appearance and deterioration occurs. As a rule the flavor is impaired, owing to the development of rancidity and off flavors.

Apparently the candy maker is unable to foretell the trouble, which does not seem to be limited to any particular type of chocolate. However, the chocolate must have a fondant center. A fondant is composed of egg white, sugar, flour, flavoring and perhaps other ingredients such as cream, butter, nuts, fruits, etc.

## EXPERIMENTAL RESULTS

From the character of the phenomenon, it seemed quite probable that it was due to fermentation. Accordingly, some preliminary tests were made to discover possible fermentative organisms. Smith fermentation tubes containing glucose bouillon and a piece of meat were employed and portions of the fondant from exploded chocolates were inoculated into them under aseptic precautions; they were then incubated at 37°C. for three days or more. Many of the tubes showed extensive gas formation.

Attempts to isolate the gas formers contained in the Smith tubes were futile until anaerobic methods were employed. The anaerobe commonly present was a spore-former of the *B. sporogenes* type. Some times the organism was present in pure culture but usually other spore-formers of the *B. subtilis* type were also present. Non-sporulating bacteria were apparently absent.

*Source of the gas-forming anaerobe*

Obviously the anaerobe causing the fermentation might come from any one of the constituents entering into the fondant. Suspicion would attach especially to the egg white rather than

to the other ingredients. The egg used by manufacturers is the dried material secured in barrel lots. Some of this egg albumen was secured and analyzed for gas-formers with results paralleling those obtained with the fondant.

In preparing the egg albumen abundant opportunity is afforded for contamination, since the eggs used are not sterilized before breaking. The shell is frequently contaminated by hen feces in the nest.

Hen feces were next tested for anaerobes. It was necessary to heat at 80°C. for ten minutes to kill off *Bact. coli* and similar forms. Anaerobes of the *B. sporogenes* type were present in feces secured from two flocks of chickens. Thus a presumptive chain of evidence attaches blame to the egg albumen as the probable source of contamination.

Before using the egg albumen it is necessary to dissolve it by soaking in water for a number of hours. This affords an excellent opportunity for the spore formers to grow and be in condition to develop gas when the fondant is prepared and coated with chocolate. The chocolate cover would retard the absorption of air, thus facilitating the growth of the anaerobes. When the fermentation of the sugar has developed sufficient gas pressure, the chocolate explodes.

At this time the work was taken up by Miss Grace A. Hill, since appointed bacteriologist in the State College at Pullman, Wash. Miss Hill repeated and extended the work very materially, and her results were embodied as part of her master's thesis (University of Washington, 1920). She analyzed five lots of exploded chocolates secured at different times from individual firms. In all, 30 trials were made involving approximately 150 chocolates. Of these 30 trials, 17 revealed gas forming bacteria, while 13 failed to show them. One of the five lots repeatedly failed to show gas-forming bacteria.

From the above analyses Miss Hill isolated a spore-forming anaerobe producing gas in sugar bouillon. All the common sugars as well as mannitol, dulcitol and inulin were fermented with gas formation. This organism we have since identified as *B. sporogenes* by agglutination and cultural studies.

Miss Hill also isolated a second gas-forming anaerobe which apparently did not form spores and which soon died out in her cultures, thus preventing identification or further study. In our earlier and later studies this organism has not been found. Yeasts were found in some of the chocolates in one lot, but since the candies were badly broken the yeasts were assumed to be possible contaminations and were not studied further. Aerobic gas-formers of the *Bact. coli* type were never found although lactose litmus agar plates were repeatedly made from the fermentation tubes.

It was noted above that one lot of cracked chocolates never gave gas-formers when inoculated into glucose Smith tubes which were freshly boiled to drive out absorbed oxygen, and which contained a piece of meat to promote anaerobiosis. The writer has since received another lot of candies which contained cherry centers and showed cracking. When tested as before, these failed to show gas-forming bacteria. The cherries taken from the barrel also failed to show gas formers. Apparently the cracking of chocolates may at times be due to non-bacterial causes, and in the above case this cause seemed quite clearly to be rough handling by the employees.

It is possible that expansion due to increased temperature may also cause cracking, but we have no evidence for this; we know, however, that normally a chocolate slowly loses its moisture content and this would tend to prevent cracking. Apparently true explosions in chocolates are always due to the accumulation of gases formed by fermentative microorganisms.

#### INOCULATION EXPERIMENTS

Thus far the evidence strongly favors the conclusion that gas-forming anaerobes cause the explosion of chocolates. To make the conclusion certain, inoculation experiments were carried out. These experiments were performed in two ways. In the first trial, various kinds of chocolates were secured, small holes drilled through the coating, and a culture of *B. sporogenes* was inoculated into the fondant through this hole by means of a

heavy platinum needle. The opening was then sealed with melted chocolate, and the samples incubated at room temperature. None of the chocolates exploded when incubated for fifteen days. It seemed probable that the chocolates used had evaporated too much of their moisture content to permit growth of the organisms.

The experiment was then repeated by inoculating the culture into the fondant just before dipping. The freshly dipped and inoculated chocolates were then carried to the laboratory and incubated at room temperature as before. On the tenth day the inoculated chocolates were found exploded, while the controls remained intact after several months. The fondant consisted of egg white, sugar, butter and ground walnuts.

The experiment was repeated by adding cultures of *Bact. coli* and *Sacch. cereviccae*. The chocolates inoculated with *Bact. coli* failed to explode after prolonged incubation, but those inoculated with the yeast as well as those inoculated with *B. sporogenes* were found exploded on the tenth day. The controls remained intact. The findings indicate that the yeasts found in one of our earlier analyses were perhaps not without significance. Apparently too, more than one type of organism is capable of producing gaseous fermentation with consequent explosion of the chocolates. We have no evidence to show that the *Bact. coli* type of organism is ever causally related to this phenomenon since it was never found in exploded chocolates, and inoculation experiments were negative.

#### SUMMARY

1. Chocolate candies are subject to explosion due to the development of gas-forming microorganisms within them. This type of explosion should be distinguished from mere cracking due to rough handling of the candies.

2. Anaerobic bacteria of the type of *B. sporogenes* are the chief cause of explosion in chocolates, but yeasts may also cause the phenomenon. Apparently the *Bact. coli* type of organisms is not involved.

3. The source of the infection has been found in the egg albumen used, and this is probably the most prevalent source, but other materials used may obviously carry the infection at times.<sup>2</sup>

<sup>2</sup> The writer desires to acknowledge with gratitude the assistance of Miss Grace A. Hill, whose help materially advanced the work; of Miss Edna Hamilton, who assisted in part of the inoculation experiments; of Mr. Fred A. Anderson, scientific expert for one of the local candy companies, for valuable information and advice; and of all the local firms which cooperated in the work without whose aid it could not have been completed.

## MICROÖRGANISMS CONCERNED IN THE OXIDATION OF SULFUR IN THE SOIL

### IV. A SOLID MEDIUM FOR THE ISOLATION AND CULTIVATION OF THIOBACILLUS THIOOXIDANS<sup>1</sup>

SELMAN A. WAKSMAN

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*Thiobacillus thiooxidans* is the organism chiefly responsible for the oxidation of sulfur in the soil, particularly when elementary sulfur has been added. This is true in ordinary soils, with the possible exception of the black alkali soils, where other organisms may also play an important part, as pointed out in the following paper. *Th. thiooxidans* is usually not originally present in the soil, but is introduced there artificially with the sulfur added. Certain soils themselves may harbor organisms which are able to oxidize small amounts of sulfur, to a much smaller extent however than *Th. thiooxidans*.

This organism has been isolated from soil-sulfur-rock phosphate composts (Lipman, Waksman and Joffe, 1921; Waksman and Joffe, 1922) by the use of liquid media only. It can exist and reproduce under very acid reactions, since it has its optimum at a pH = 3.0 to 4.0, and can also grow at as low a pH as 1.0. It derives its energy from the oxidation of inorganic, elementary sulfur, and its carbon from the CO<sub>2</sub> of the atmosphere, while its nitrogen need is obtained from ammonium salts or nitrates and its mineral need from traces of potassium, magnesium and iron salts and phosphates. By the use of media containing such substances, and by employing high dilutions the organism was isolated in pure culture. The purity of the culture was established by the following facts: the organism grew readily upon the inorganic liquid media, giving always the same uniform turbidity;

<sup>1</sup> Paper no. 84 of the Journal Series, New Jersey Agricultural Experiment Station, Department of Soil Chemistry and Bacteriology.

it did not grow in bouillon, on nutrient agar, or other common organic and inorganic media, favorable for the cultivation of bacteria and fungi; it gave a uniform microscopic picture, both in unstained and stained preparations. The culture was kept on liquid media, for over one and one-half years by transferring it to fresh lots of media every one to four weeks. All attempts to develop a solid medium for the cultivation of the organism failed until December of last year.

A detailed study of the media commonly used for the cultivation of colorless sulfur bacteria not accumulating sulfur within their cells is given elsewhere (Waksman, 1922). The two liquid media best adapted for the isolation and cultivation of *Thiobacillus thiooxidans* have the following composition:

I	II
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> .....0.2 gram	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ..... 0.2 gram
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....0.5 gram	MgSO <sub>4</sub> ·7H <sub>2</sub> O..... 0.5 gram
KH <sub>2</sub> PO <sub>4</sub> .....3.0 grams	KH <sub>2</sub> PO <sub>4</sub> ..... 1.0 gram
CaCl <sub>2</sub> .....0.25 gram	Re-precipitated Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub> 2.5 grams
Elementary, powdered	Sulfur..... 10.0 grams
sulfur..... 10 grams	H <sub>3</sub> PO <sub>4</sub> $\left(\frac{M}{1}\right)$ to adjust reaction to
Distilled water.....1000 cc.	pH = 3.0
	Distilled water.....1000 cc.

The sulfur in both media and the Ca<sub>3</sub>PO<sub>4</sub> in medium II are weighed out separately in the individual flasks into which the media are distributed (100-cc. portions are usually placed in 250-cc. flasks). The media are sterilized for thirty minutes, in flowing steam, on three consecutive days.

When a flask with one of these two media is inoculated from a fresh vigorous culture (seven to fourteen days old) of the *Th. thiooxidans* growth will be manifested by a uniform turbidity, without any pellicle formation, within four to five days, at 25 to 30°C., the culture becoming very turbid in seven to eight days. The same phenomenon is observed when the medium is inoculated with a little soil containing the organisms, only the length of time required for development is sometimes a little longer, depending upon the abundance of the organism in the soil, condition of soil, etc. By using the dilution method, even the



approximate number of the organisms in the soil can be estimated. The culture obtained on the two media is practically pure, due to the fact that very few other organisms would develop under these conditions. However, both for the purpose of establishing the purity of the culture and for the better characterization of the organism, the development of a solid medium was extremely desirable.

Among the various solid media tried, without any success, for the cultivation of *Th. thiooxidans* was Beijerinck's thiosulfate medium (1904) upon which *Th. thioparus* grew readily. Although *Th. thiooxidans* can utilize thiosulfate as a source of energy, it did not grow on this medium due to the extreme alkalinity of that medium and to the lack of a calcium salt. When the  $\text{NaHCO}_3$  is eliminated from this medium, the dibasic potassium salt changed to the monobasic form and when calcium chloride is added, a solid medium favorable for the growth of *Th. thiooxidans* is obtained.

The composition of the medium is as follows:

$\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ .....	5.0	grams
$\text{KH}_2\text{PO}_4$ .....	3.0	grams
$\text{NH}_4\text{Cl}$ .....	0.1	gram
$\text{MgCl}_2$ .....	0.1	gram
$\text{CaCl}_2$ .....	0.25	gram
Agar.....	20.0	grams
Distilled water.....	1000	cc.

The medium is prepared as usual and sterilized at 15 pounds pressure for fifteen minutes. Plates and slants are inoculated from a vigorous liquid culture and incubated at 25 to 30°C. Growth appears in five to six days in the form of minute straw yellow to cream-colored colonies. Under the microscope, each colony is surrounded with crystals of gypsum due to the action of the sulfuric acid, formed from the oxidation of the thiosulfate, upon the  $\text{CaCl}_2$ . This phenomenon is particularly prominent in media containing tri-calcium phosphate in place of the chloride; a clear zone is formed around each colony, due to the disappearance of the insoluble calcium salt.

*Th. thioparus* practically makes no growth on this medium, due to its acid reaction. In the original Beijerinck medium, however, containing dibasic phosphate and  $\text{NaHCO}_3$ , *Th. thiooxidans* makes no growth, while *Th. thioparus* grows in the form of minute pinpoint colonies covered with sulfur, which is separated from the thiosulfate by the action of the latter organism. The *Th. thiooxidans* separates no sulfur, oxidizing the thiosulfate completely to sulfate; *Th. thioparus* oxidizes the thiosulfate to sulfate, persulfates and elementary sulfur.

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## MICROÖRGANISMS CONCERNED IN THE OXIDATION OF SULFUR IN THE SOIL

### V. BACTERIA OXIDIZING SULFUR UNDER ACID AND ALKALINE CONDITIONS<sup>1</sup>

SELMAN A. WAKSMAN

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A detailed description of media and methods of isolation of sulfur bacteria concerned in the oxidation of sulfur in the soil has been given in the previous two papers of this series (Waksman, 1922 a, 1922 b).

The work reported in this paper is limited to two liquid (nos. 8 and 6) and two solid media (nos. 9 and 10). Medium 8 consists of 5 grams  $\text{Na}_2\text{S}_2\text{O}_3$ , 0.1 gram  $\text{NH}_4\text{Cl}$ , 0.1 gram  $\text{MgCl}_2$ , 0.2 gram  $\text{Na}_2\text{HPO}_4$  (or  $\text{K}_2\text{HPO}_4$ ), 1 gram  $\text{NaHCO}_3$  and 10 grams  $\text{CaCO}_3$  or 0.25 gram  $\text{CaCl}_2$  dissolved in 1000 cc. of tap water. This is only a slight modification of the original Beijerinck (1904) medium. Medium 9 is the same as 8, but with 2 per cent of agar added. These media are sterilized, at 15 pounds pressure, for fifteen minutes; the thiosulfate and carbonate are sterilized, in the case of the liquid media, separately, then added by means of sterile pipettes. The initial reaction of these media is about  $\text{pH} = 8.6$  to  $9.6$ . Medium 6 consists of 0.2 gram  $(\text{NH}_4)_2\text{SO}_4$ , 0.5 gram  $\text{MgSO}_4$ , 0.25 gram  $\text{CaCl}_2$ , 3 grams  $\text{KH}_2\text{PO}_4$ , trace of  $\text{FeSO}_4$  and 10 grams of sulfur in 1000 cc. of distilled water. This medium is sterilized in flowing steam, for thirty minutes on three consecutive days. Its initial reaction is  $\text{pH} = 4.0$  to  $4.8$ . Medium 10 consists of 5 grams  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ , 0.1 grams  $\text{NH}_4\text{Cl}$ , 0.1 gram  $\text{MgCl}_2$ , 0.25 gram  $\text{CaCl}_2$  (or 10 grams  $\text{Ca}_4(\text{PO}_4)_2$ ), 3 grams  $\text{KH}_2\text{PO}_4$ , 20 grams of agar in 1000

<sup>1</sup> Paper no. 85 of the Journal Series, New Jersey Agricultural Experiment Stations, Department of Soil Chemistry and Bacteriology.

cc. of distilled water. This medium is sterilized in the autoclave, at 15 pounds for fifteen minutes.

When a small particle of soil is added to media 6 and 9, growth may or may not take place according to the nature of the soil, the two media behaving in a distinctly different way. Medium 6 is acid in reaction and contains elementary sulfur as the only source of energy; only those organisms which can oxidize elementary sulfur and can live at a pH of 4.0 and less will develop in this medium. Such organisms are not usually present in common cultivated soils, unless the soils have had previous applications of elementary sulfur. The organism developing on this medium is the *Th. thiooxidans* Waksman and Joffe (1922) described in detail elsewhere. This organism is not universally present in the soil, but is present in soil mixed with powdered sulfur, as around the sulfur mines, etc. This is the reason why the organism was originally isolated from composts of sulfur with soil or sulfur, rock phosphate and soil. This organism oxidizes sulfur, in pure culture, quantitatively to sulfuric acid; it also oxidizes thiosulfate, without the separation of elementary sulfur, with active acid formation. The growth of this organism on the liquid media is quite characteristic: it forms, in four to five days, at 25°C., a turbidity throughout the medium, without any pellicle formation. In the presence of calcium salts, crystals of gypsum soon appear, both on the bottom of the flask and hanging down from the particles of sulfur. The sulfur floating on the surface of the medium drops to the bottom. When transferred to medium 10, minute pale to pale-yellow to dirty-yellow (or light cream colored) colonies appear in five to six days. In the presence of insoluble calcium salts, like the carbonate and the phosphate, a colorless halo is rapidly formed around each colony on the plate, due to the action of the sulfuric acid formed from the oxidation of the thiosulfate upon the insoluble calcium salts. A ring of gypsum crystals is formed around each colony.

Medium 8 behaves differently; organisms that can thrive at distinctly alkaline reactions and that can oxidize thiosulfate will develop on this medium. Such organisms are universally dis-

tributed in the soil. Whether these organism form a group of closely related strains or whether they form a group of organisms differing in morphological and physiological characteristics still remains to be seen. The characteristic growth of this group of organisms is as follows: when a piece of soil is added to medium 8 (better growth will take place, when the medium is not sterilized), and flasks incubated at 25°C., growth will appear in forty-eight to seventy-two hours. This growth consists of a pale colored pellicle formed on the surface of the medium and on the glass of the flask; the medium does not become turbid, unless *Th. thiooxidans* is also present in the soil. An abundant separation of the sulfur from the thiosulfate takes place, so that the surface pellicle becomes sulfur-yellow. The thiosulfate rapidly disappears with the formation of sulfates, persulfates and elementary sulfur. If more thiosulfate is added to the flask, it will be further transformed, so that 5 grams of thiosulfate may be transformed in a 100-cc. culture in two to three weeks; this will take place thus rapidly, however, only when a proportionate amount of  $\text{NaHCO}_3$  is added with the thiosulfate (1:5). The rôle of the bicarbonate consists in supplying the base to the formation of the sulfate and keeping the medium from becoming acid. Whether the bicarbonate may also serve, at least partly, as a source of carbon, is still unknown; however, it does not do so for *Th. thiooxidans*. The reaction of the medium becomes only slightly acid. Where intense acid formation accompanied by the breaking up of the sulfur pellicle has been found, *Th. thiooxidans* has invariably been demonstrated to be present. When a streak is made from the culture in medium 8 upon a plate containing cooled medium 9, minute colonies will rapidly develop, characterized by an abundant separation of sulfur, which covers the whole colony. No clear zone, or a mere trace of it, is formed around the colony, unless *Th. thiooxidans* is also present. This organism thus developing on medium 8 is closely related in its physiological characteristics to the *Th. thioparus* of Beijerinck, which was demonstrated by Gehring (1915) to be universally distributed in the soil. This organism will be called *Thiobacillus B*. This organism can be cultivated very readily,

but rapidly deteriorates on artificial culture media. Its absolute purity, particularly as far as contamination with *Th. thiooxidans* or a modified strain of this organism was not established as yet.

When sulfur is added to a neutral or acid soil, it is rapidly oxidized by *Th. thiooxidans* when present or introduced. When sulfur is added to an alkali soil, particularly a black alkali soil, the oxidation is very slow, even in the presence of *Th. thiooxidans*. However, after a little time, the sulfur is oxidized. When some of this black alkali soil, to which sulfur has been added, is introduced into flasks with media 6 and 8, an excellent growth will take place in the latter; while, in the former, growth will take place only if the oxidation is at an advanced stage. A series of studies has led the author to conclude that, while, in acid soils, the sulfur is oxidized primarily by *Th. thiooxidans*, in alkali soils *Thiobacillus B.* also takes an active part in the process. This part consists either in a direct oxidation of the sulfur, whereby the reaction of the soil is made sufficiently acid so that *Th. thiooxidans* (if present) may oxidize the sulfur further, or by a certain modification of the sulfur, so that it is more readily acted upon by *Th. thiooxidans* under alkaline conditions. The chemistry of the process of sulfur oxidation both in acid and alkali soils by the two organisms has been reported in detail elsewhere (Waksman and Joffe, 1921, Waksman, 1922 c).

The following three tables show clearly the relation between initial reaction, source of sulfur and sulfur oxidation.

The following tables definitely establish the fact that *Th. thiooxidans* primarily oxidizes elementary sulfur under acid conditions. The possibility that it may oxidize elementary sulfur in the soil, under alkaline conditions is thereby not excluded, particularly in the presence of other organisms. It also oxidizes thiosulfate with an intense acid formation. *Thiobacillus B.* oxidizes very little elementary sulfur in the soil or in solution, but the fact that it can act upon sulfur under highly alkaline conditions suggests the possibility of the associative action of the two organisms in changing the reaction of black alkali soil to which sulfur has been added. *Thiobacillus B.* oxidizes thiosulfate

TABLE I  
*The oxidation of elementary sulfur at different reactions*  
 Medium 6 without the  $\text{KH}_2\text{PO}_4$  used as a basis

COMPOSITION	CULTURE	pH VALUE		
		Control	6 days	21 days
(A) 3 grams $\text{KH}_2\text{PO}_4$	<i>Th. thiooxidans</i>	4.2	1.6	1.2
(B) $1\frac{1}{2}$ grams $\text{KH}_2\text{PO}_4$ + 1 $\frac{1}{2}$ grams $\text{K}_2\text{HPO}_4$	<i>Th. thiooxidans</i>	5.4	3.0	1.2
(C) 3 grams $\text{K}_2\text{HPO}_4$	<i>Th. thiooxidans</i>	6.0	5.4	1.4
(D) 3 grams $\text{K}_2\text{HPO}_4$ + 10 grams $\text{CaCO}_3$	<i>Th. thiooxidans</i>	8.0	8.0	8.0
(A)	<i>Thiobacillus B.</i>	4.2	3.8	3.6
(B)	<i>Thiobacillus B.</i>	5.4	3.8	3.8
(C)	<i>Thiobacillus B.</i>	6.0	4.0	3.8
(D)	<i>Thiobacillus B.</i>	8.0	8.0	8.0
(A)	<i>Th. thiooxidans</i> + <i>Th. B.</i>	4.2	2.0	1.2
(B)	<i>Th. thiooxidans</i> + <i>Th. B.</i>	5.4	2.2	1.4
(C)	<i>Th. thiooxidans</i> + <i>Th. B.</i>	6.0	1.8	1.4
(D)	<i>Th. thiooxidans</i> + <i>Th. B.</i>	8.0	8.0	6.4
(A)	Ordinary soil	4.2	4.2	4.2
(B)	Ordinary soil	5.4	5.4	5.2
(C)	Ordinary soil	6.0	6.0	5.4
(D)	Ordinary soil	8.0	8.0	7.6
(A)	Acid compost (soil + rock phosphate + sulfur)	4.2	2.6	1.4
(B)	Acid compost	5.4	3.4	1.4
(C)	Acid compost	6.0	5.0	1.8
(D)	Acid compost	8.0	8.0	7.6
(A)	Alkali compost (alkali soil + sulfur)	4.2	4.2	4.2
(B)	Alkali compost	5.4	3.8	3.8
(C)	Alkali compost	6.0	4.6	4.4
(D)	Alkali compost	8.0	8.0	6.4

rapidly, with an abundant separation of sulfur. This separation of sulfur from the thiosulfate as a result of the action of this organism indicates its rather weak sulfur-oxidizing power. When *Th. thiooxidans* is also present, the pellicle is either not formed at all, especially when the medium is acid, or, in alkaline

TABLE 2  
*The oxidation of thiosulfate at different reactions*  
 Medium S used as a basis, without any carbonate

COMPOSITION	CULTURE	pH VALUE		
		Control	6 days	21 days
(A) 3 grams $\text{KH}_2\text{PO}_4$	<i>Th. thiooxidans</i>	5.4	4.4	1.4
(B) Medium S, without carbonate	<i>Th. thiooxidans</i>	6.2	5.0	1.6
(C) 1 gram $\text{NaHCO}_3$	<i>Th. thiooxidans</i>	8.8	8.8	8.8
(D) 1 gram $\text{NaHCO}_3$ + 10 grams $\text{CaCO}_3$	<i>Th. thiooxidans</i>	9.4	9.4	9.4
(A)	<i>Thiobacillus B.</i>	5.4	5.4	5.4
(B)	<i>Thiobacillus B.</i>	6.2	6.2	6.0
(C)	<i>Thiobacillus B.</i>	8.8	6.2	6.4
(D)	<i>Thiobacillus B.</i>	9.4	6.4	7.0
(A)	<i>Th. thiooxidans</i> + <i>Th. B.</i>	5.4	4.8	1.4
(B)	<i>Th. thiooxidans</i> + <i>Th. B.</i>	6.2	5.2	1.4
(C)	<i>Th. thiooxidans</i> + <i>Th. B.</i>	8.8	5.2	1.6
(D)	<i>Th. thiooxidans</i> + <i>Th. B.</i>	9.4	5.8	6.6
(A)	Ordinary soil	5.4	5.4	5.0
(B)	Ordinary soil	6.2	6.2	5.6
(C)	Ordinary soil	8.8	8.0	6.8
(D)	Ordinary soil	9.4	9.4	8.0
(A)	Acid compost	5.4	5.4	5.0
(B)	Acid compost	6.2	6.2	5.4
(C)	Acid compost	8.8	8.4	6.0
(D)	Acid compost	9.4	9.4	8.0
(A)	Alkali compost	5.4	5.2	5.0
(B)	Alkali compost	6.2	6.4	5.6
(C)	Alkali compost	8.8	8.6	5.8
(D)	Alkali compost	9.4	9.4	7.6

media, it is rapidly broken up as a result of the rapid oxidation of the sulfur to sulfuric acid by *Th. thiooxidans*.

In the study of ordinary cultivated soil, the presence of *Thiobacillus B.* or organisms closely related in their metabolism is easily demonstrated. The acid compost, which is a mixture of sulfur (5 per cent), rock phosphate (15 per cent) and soil,



inoculated with crude cultures of *Th. thiooxidans* has become so acid due to long incubation (one year) that only the latter organism survives. If *Thiobacillus B.* was originally in the compost, it has died out, due to the intense acidity. The alkali compost consisting of black alkali, to which 0.5 per cent of sulfur has been added and incubated for 6 months, definitely indicates the presence of the second organism.

The sulfide (table 3) was oxidized to some extent by both organisms, and particularly by a mixture of the two organisms. This is explained by the fact that the sulfur produced from the sulfide by the action of *Th. B.* is rapidly oxidized to sulfuric acid

TABLE 3  
*The oxidation of K<sub>2</sub>S by microorganisms*

Medium 8 used as a basis, 0.5 per cent K<sub>2</sub>S in place of the thiosulfate

CULTURE	pH VALUE			SULFATES, MILLIGRAMS OF S IN 100 CC.
	Control	6 days	21 days	
<i>Th. thiooxidans</i> *.....	7.5	7.5	7.5	14.11
<i>Thiobacillus B.</i> .....	7.5	5.8	4.5	43.84
<i>Th. thiooxidans</i> + <i>Thiobacillus B.</i> .....	7.5	5.2	1.4	90.42

\* In some cases, particularly at lower pH values, the oxidation of the sulfide by the *Th. thiooxidans* was much more extensive.

by *Th. thiooxidans*. This is well demonstrated by the column of sulfates formed. A detailed study of the morphology of these two organisms will form the subject of a succeeding paper.

#### SUMMARY

1. At least two organisms can be shown to take part in the oxidation of sulfur in the soil; while, in acid soils, *Th. thiooxidans* will rapidly oxidize the elementary sulfur, in alkali soils, particularly black alkali soils, still another organism, *Thiobacillus B.*, similar to *Th. thioparus* of Beijerinck, takes an active part in this process.

2. *Th. thiooxidans* is usually not present in common cultivated soils, but is found abundantly in soils previously treated with sulfur. *Thiobacillus B.* is commonly present in cultivated soils.

3. By the interaction of the two organisms, sulfur can be oxidized at reactions ranging from pH = 9.8 to 1.0.

TAXONOMIC POSITION OF THE COLORLESS SULFUR OXIDIZING  
ORGANISMS NOT ACCUMULATING SULFUR WITHIN  
THEIR CELLS

The colorless organisms oxidizing sulfur and sulfur compounds and not accumulating sulfur within their cells have been classified by Beijerinck (1904) under the genus *Thiobacillus*. Orla-Jensen (1909) properly indicated the relationships of these organisms both in their morphology and physiology to the nitrifying bacteria, methane, hydrogen and other autotrophic organisms, and suggested the generic name *Sulfomonas* in place of *Thiobacillus*. In view of the fact that the Committee on Characterization and Classification of the S. A. B. (Winslow et al., 1920) has adapted several of the new generic names suggested by Orla-Jensen for the members of the Nitrobacterae, the generic name *Sulfomonas* should be used rather than that of *Thiobacillus*, and this genus of sulfur oxidizing bacteria should be classified with the Nitrobacterae. This genus will now consists of three species:

*Sulfomonas thioparus* (syn. *Thiobacillus thioparus* Beijerinck, Orla-Jensen 1909, p. 314).

*Sulfomonas denitrificans* (syn. *Thiobacillus denitrificans* Beijerinck, Orla-Jensen 1909, p. 314).

*Sulfomonas thiooxidans* (syn. *Thiobacillus thiooxidans*, Waksman and Joffe).

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## INDEX TO VOLUME VII

Aerobic spore-bearing bacteria, Influence of vacuum upon growth of some . . .	283
— thermophilic bacteria from water. Studies on thermophilic bacteria, I, . . .	343
Agar slants, The use of, in detecting ammonia production and its relation to the reduction of nitrates. . . . .	515
Agglutination, spontaneous, A study of, in the colon-typhoid group of bacilli. . . . .	71
—, Studies upon, in the colon-typhoid group of bacilli. . . . .	39
Albus, W. R., Sherman, J. M. and Holm, G. E. Salt effects in bacterial growth. III. Salt effects in relation to the lag period and velocity of growth. . . . .	583
American gentian violets, An investigation of. Report of committee on bacteriological technic. . . . .	529
— stains, An investigation of. Report of committee on bacteriological technic. . . . .	127
Ammonia production and its relation to the reduction of nitrates, The use of agar slants in detecting. . . . .	515
Anaerobe, saprophytic, Quantitative determination of some of the biochemical changes produced by a. . . . .	373
Anaerobes, A method for the cultivation of. . . . .	277
—, pathogenic, Studies in, V. Certain genera of the clostridiaceae. . . . .	1
Apparatus, An, for the rapid measurement of surface tension. . . . .	367
Autolysis, Bacterial. . . . .	551
Ayers, S. Henry and Mudge, Courtland S. The relation of vitamins to the growth of a streptococcus. . . . .	449
<i>B. putrificus</i> Bienstock, <i>Clostridium putrificum</i> , a distinct species. . . . .	505
Bacilli, A study of spontaneous agglutination in the colon-typhoid group of . . . . .	71
Bacilli, Studies upon agglutination in the colon-typhoid group of. . . . .	39
<i>Bacillus hemoglobinophilus canis</i> (Friedberger) ( <i>Hemophilus canis emend</i> ).. . . .	579
<i>Bact. coli</i> , The effects of temperature and hydrogen ion concentration upon the viability of, and <i>Bact. typhosum</i> in water. Disinfection studies. . . . .	183
—, The growth of, in relation to H-ion concentration. Salt effects in bacterial growth, II. . . . .	465
<i>Bact. typhosum</i> in water. The effects of temperature and hydrogen ion concentration upon the viability of <i>Bact. coli</i> and. Disinfection studies . . . . .	183
Bacteria, A binocular microscope arranged for the study of colonies of. . . . .	121
—, Influence of vacuum upon growth of some spore-bearing. . . . .	283
—, Lactic acid, Studies on the biology of, a summary of personal investigations. . . . .	271
—, Method for the isolation of, in pure culture from single cells and procedure for the direct tracing of bacterial growth on a solid medium. . . . .	537

Bacteria, A method of detecting rennet production by.....	447
— oxidizing sulfur under acid and alkaline conditions. Microorganisms concerned in the oxidation of sulfur in the soil, V.....	609
—, The proportion of viable, in young cultures with especial reference to the technique employed in counting.....	405
—, The sources and characteristics of the, in decomposing salmon.....	85
—, Studies on cultural requirements of, I.....	309
—, Studies on cultural requirements of, II.....	325
— symbiotic in the tissues of higher organisms, A note on the morphology of.....	471
—, thermophilic, Studies on, I. Aerobic thermophilic bacteria from water.	343
Bacterial autolysis.....	551
— growth on a solid medium, procedure for the direct tracing of, Method for the isolation of bacteria in pure culture from single cells and.....	537
— —, Salt effects in, II. The growth of <i>Bact. coli</i> in relation to H-ion concentration.....	465
— —, — — —, III. Salt effects in relation to the lag period and velocity of growth.....	583
Bacteriological medium, Transparent milk as a.....	511
Bacteriolysants, Observations on the nature of (D'Herelle's phenomenon, bacteriophage, bacteriolytic agent, etc.).....	491
—, Observations on the properties of (D'Herelle's phenomenon, bacteriophage, bacteriolytic agent, etc.).....	475
Bacteriolytic agent, etc. D'Herelle's phenomenon, bacteriophage, observations on the nature of bacteriolysants.....	491
Bacteriophage, bacteriolytic agent, etc., D'Herelle's phenomenon, Observations on the nature of bacteriolysants.....	491
Baker, H. R. Substitution of brom-thymol-blue for litmus in routine laboratory work.....	301
Beverages, carbonated, Viability of the colon-typhoid group in carbonated water and.....	111
Binocular, A, microscope arranged for the study of colonies of bacteria....	121
Biochemical changes produced by a saprophytic anaerobe. Quantitative determination of some of the.....	373
Biology, Studies on the, of lactic acid bacteria: a summary of personal investigations.....	271
Breed method, The use of domestic methylene blue in staining milk by the..	307
Brom-thymol-blue, Substitution of, for litmus in routine laboratory work..	301
Brown, J. Howard and Howe, Paul E. Transparent milk as a bacteriological medium.....	511
Burke, Victor. Notes on the Gram stain with description of a new method..	159
Bushnell, L. D. Influence of vacuum upon growth of some aerobic spore-bearing bacteria.....	283
Bushnell, L. D. A method for the cultivation of anaerobes.....	277
— Quantitative determination of some of the biochemical changes produced by a saprophytic anaerobe.....	373

Candies, chocolate, The cause of explosion in .....	599
Carbonated water and carbonated beverages, Viability of the colon-typhoid group in.....	111
Cause, The, of explosion in chocolate candies.....	599
Certain genera of the clostridiaceae. Studies in pathogenic anaerobes, V..	I
Chocolate candies, The cause of explosion in.....	599
Clostridiaceae, Certain genera of the. Studies in pathogenic anaerobes, V..	I
<i>Clostridium putrificum</i> ( <i>B. putrificus</i> Bienstock), a distinct species.....	505
Cohen, Barnett. Disinfection studies. The effects of temperature and hydrogen ion concentration upon the viability of <i>Bact. coli</i> and <i>Bact. typhosum</i> in water.....	183
Colon-typhoid group in carbonated water and carbonated beverages, Viability of the.....	111
Colon-typhoid group of bacilli, A study of spontaneous agglutination in the.	71
Colon-typhoid group of bacilli, Studies upon agglutination in the.....	39
Colonies of bacteria, A binocular microscope arranged for the study of ...	121
"Color standards" for the colorimetric determination of H-ion concentration, Further observations on.....	589
Colorimetric determination of H-ion concentration, Further observations on "color standards" for the.....	589
Committee on bacteriological technic, Report of. An investigation of American gentian violets.....	529
— on bacteriological technic, Report of. An investigation of American stains.....	127
— on bacteriological technic, Report of. Methods of pure culture study.	519
Conn, H. J., Chairman. An investigation of American gentian violets. Report of committee on bacteriological technic.....	529
— — An investigation of American stains. Report of the committee on bacteriological technic.....	127
— — Methods of pure culture study. Report of committee on bacteriological technic.....	519
Conn, H. J. A method of detecting rennet production by bacteria.....	447
Counting the number of fungi in the soil, A method for.....	339
—, The proportion of viable bacteria in young cultures with especial reference to the technique employed in.....	405
Cultivation of anaerobes, A method for the.....	277
— of <i>Thiobacillus thiooxidans</i> , a solid medium for the isolation and. Microorganisms concerned in the oxidation of sulfur in the soil, IV.....	605
Cultural requirements of bacteria, Studies on, I.....	309
— — — — —, Studies on, II.....	325
Culture study, Methods of pure. Report of committee on bacteriological technic.....	519
Davison, Wilburt C. Observations on the nature of bacteriolyants (D'Herelle's phenomenon, bacteriophage, bacteriolytic agent, etc.)	491
— Observations on the properties of bacteriolyants (D'Herelle's phenomenon, bacteriophage, bacteriolytic agent, etc.).....	475
D'Herelle's phenomenon, bacteriophage, bacteriolytic agent, etc., Observations on the nature of bacteriolyants.....	491

Disinfection studies. The effects of temperature and hydrogen ion concentration upon the viability of <i>Bact. coli</i> and <i>Bact. typhosum</i> in water....	183
Explosion, the cause of, in chocolate candies.....	599
Fred, E. B. and Peterson, W. II. The production of pink sauerkraut by yeasts.....	257
Fungi in the soil, A method for counting the number of.....	339
Further observations on "color standards" for the colorimetric determination of H-ion concentration.....	589
Genera, Certain, of the clostridiaceae. Studies in pathogenic anaerobes, V.	1
Gentian violets, An investigation of American. Report of committee on bacteriological technic.....	529
Gorini, Costantino. Studies on the biology of lactic acid bacteria: a summary of personal investigations.....	271
Gram stain, Notes on the, with description of a new method.....	159
Green, Robert G. An apparatus for the rapid measurement of surface tension.....	367
Growth, bacterial, on a solid medium, procedure for the direct tracing of, Method for the isolation of bacteria in pure culture from single cells and	537
—, —, Salt effects in, III. Salt effects in relation to the lag period and velocity of growth.....	583
— of a streptococcus, The relation of vitamins to the.....	449
— of some aerobic spore-bearing bacteria, Influence of vacuum upon....	283
—, Salt effects in relation to the lag period and velocity of. Salt effects in bacterial growth, III.....	583
—, The, of <i>Bact. coli</i> in relation to H-ion concentration. Salt effects in bacterial growth, II.....	465
H-ion concentration, Further observations on "color standards" for the colorimetric determination of.....	589
— —, The growth of <i>Bact. coli</i> in relation to. Salt effects in bacterial growth, II.....	465
Harrison, F. C. Our society.....	149
Heller, Hilda Hempl. Certain genera of the clostridiaceae. Studies in pathogenic anaerobes, V.....	1
<i>Hemoglobinophilus canis</i> , <i>Bacillus</i> (Friedberger) ( <i>Hemophilus canis emend</i> )..	579
<i>Hemophilus canis emend</i> , <i>Bacillus hemoglobinophilus canis</i> (Friedberger)...	579
Holm, George E. and Sherman, James M. Salt effects in bacterial growth. II. The growth of <i>Bact. coli</i> in relation to H-ion concentration.....	465
—, —, and Albus, W. R. Salt effects in bacterial growth. III. Salt effects in relation to the lag period and velocity of growth.....	583
Howe, Paul E. and Brown, J. Howard. Transparent milk as a bacteriological medium.....	511
Hucker, G. J. and Wall, W. A. The use of agar slants in detecting ammonia production and its relation to the reduction of nitrates.....	515
Hunter, Albert C. The sources and characteristics of the bacteria in decomposing salmon.....	85
Hydrogen ion concentration, The effects of temperature and, upon the viability of <i>Bact. coli</i> and <i>Bact. typhosum</i> in water. Disinfection studies..	183

Influence of vacuum upon growth of some anaerobic spore-bearing bacteria..	283
Investigation, An, of American geutian violets. Report of committee on bacteriological technic.....	529
—, —, of American stains. Report of committee on bacteriological technic.....	127
Ishii, O. A study of spontaneous agglutination in the colon-typhoid group of bacilli.....	71
— Studies upon agglutination in the colon-typhoid group of bacilli . . .	39
Isolation and cultivation of <i>Thiobacillus thiooxidans</i> , A solid medium for the. Microorganisms concerned in the oxidation of sulfur in the soil, IV....	605
— of bacteria in pure culture from single cells, Method for the, and procedure for the direct tracing of bacterial growth on a solid medium.....	537
Joffe, J. S. and Waksman, Selman A. Microörganisms concerned in the oxidation of sulfur in the soil II. <i>Thiobacillus thiooxidans</i> , a new sulfur-oxidizing organism isolated from the soil.....	239
Koser, S. A. and Skinner, W. W. Viability of the colon-typhoid group in carbonated water and carbonated beverages.....	111
Lactic acid bacteria: Studies on the biology of a summary of personal investigations.....	271
Lag period, Salt effects in relation to the, and velocity of growth. Salt effects in bacterial growth, III.....	583
Litmus, Substitution of brom-thymol-blue for, in routine laboratory work..	301
Medalia, L. S. Further observations on "color standards" for the colorimetric determination of H-ion concentration.....	589
Medium, bacteriological, Transparent milk as a.....	511
—, procedure for the direct tracing of bacterial growth on a solid, Method for the isolation of bacteria in pure culture from single cells and.....	537
—, A solid, for the isolation and cultivation of <i>Thiobacillus thiooxidans</i> . Microorganisms concerned in the oxidation of sulfur in the soil, IV....	605
Method, A, for counting the number of fungi in the soil.....	339
—, A, for the cultivation of anaerobes.....	277
— for the isolation of bacteria in pure culture from single cells and procedure for the direct tracing of bacterial growth on a solid medium.....	537
Method, A, of detecting rennet production by bacteria.....	447
Methods of pure culture study. Report of committee on bacteriological technic.....	519
Methylene blue, The use of domestic, in staining milk by the Breed method..	307
Microörganisms concerned in the oxidation of sulfur in the soil. I. Introductory.....	231
— — — — —. II. <i>Thiobacillus thiooxidans</i> , a new sulfur-oxidizing organism isolated from the soil.....	239
— — — — —. IV. A solid medium for the isolation and cultivation of <i>Thiobacillus thiooxidans</i> .....	605
— — — — —. V. Bacteria oxidizing sulfur under acid and alkaline conditions.....	609

Microscope, A binocular, arranged for the study of colonies of bacteria....	121
Milk, Transparent, as a bacteriological medium.....	511
—, The use of domestic methylene blue in staining, by the Breed method..	307
Morphology, A note on the, of bacteria symbiotic in the tissues of higher organisms.....	471
Morrison, Lethe E. and Tanner, Fred W. Studies on thermophilic bacteria. I. Aerobic thermophilic bacteria from water.....	343
Mudge, Courtland S. and Ayers, S. Henry. The relation of vitamins to the growth of a streptococcus.....	449
Mueller, J. Howard. Studies on cultural requirements of bacteria, I.....	309
— Studies on cultural requirements of bacteria, II.....	325
Nature of bacteriolysants (D'Herelle's phenomenon, bacteriophage, bacteriolytic agent, etc.).....	491
Nitrates, The use of agar slants in detecting ammonia production and its relation to the reduction of.....	515
Note, A, on the morphology of bacteria symbiotic in the tissues of higher organisms.....	471
Notes on the Gram stain with description of a new method.....	159
Observations on the nature of bacteriolysants (D'Herelle's phenomenon, bacteriophage, bacteriolytic agent, etc.).....	491
— on the properties of bacteriolysants (D'Herelle's phenomenon, bacteriophage, bacteriolytic agent, etc.).....	475
Ørskov, J. Method for the isolation of bacteria in pure culture from single cells and procedure for the direct tracing of bacterial growth on a solid medium.....	537
Our society.....	149
Oxidation of sulfur in the soil. Microorganisms concerned in the. I. Introductory.....	231
— — — — —. II. <i>Thiobacillus thiooxidans</i> , a new sulfur-oxidizing organism isolated from the soil.....	239
— — — — —. IV. A solid medium for the isolation and cultivation of <i>Thiobacillus thiooxidans</i> .....	605
— — — — —. V. Bacteria oxidizing sulfur under acid and alkaline conditions.....	609
Pathogenic anaerobes, Studies in, V. Certain genera of the clostridiaceae..	1
Peterson, W. H. and Fred, E. B. The production of pink sauerkraut by yeasts .....	257
Pink sauerkraut, The production of, by yeasts.....	257
Production, The, of pink sauerkraut by yeasts.....	257
Properties of bacteriolysants, Observations on the (D'Herelle's phenomenon, bacteriophage, bacteriolytic agent, etc.).....	475
Proportion, The, of viable bacteria in young cultures with especial reference to the technique employed in counting.....	405
Pure culture from single cells, Method for the isolation of bacteria in, and procedure for the direct tracing of bacterial growth on a solid medium. 537	
— — study, Methods of. Report of committee on bacteriological technic	519



Quantitative determination of some of the biochemical changes produced by a saprophytic anaerobe.....	373
Rapid measurement of surface tension, An apparatus for the.....	367
Reddish, George F. and Rettger, Leo F. <i>Clostridium putrificum</i> ( <i>B. putrificus</i> Bienstock), a distinct species.....	505
Reduction of nitrates, The use of agar slants in detecting ammonia production and its relation to the.....	515
Reed, Guilford B. A binocular microscope arranged for the study of colonies of bacteria.....	121
Relation, The, of vitamins to the growth of a streptococcus.....	449
Rennet production, A method of detecting, by bacteria.....	447
Report of committee on bacteriological technic. An investigation of American gentian violets.....	529
———, ———, ———, ———, ———, ———. An investigation of American stains.....	127
———, ———, ———, ———, ———, ———. Method of pure culture study.....	519
Rettger, Leo F. and Reddish, George F. <i>Clostridium putrificum</i> ( <i>B. putrificus</i> Bienstock), a distinct species.....	505
—— and Sturges, William S. Bacterial autolysis.....	551
Rivers, T. M. <i>Bacillus hemoglobinophilus canis</i> (Friedberger) ( <i>Hemophilus canis emend</i> ).....	579
Robertson, A. H. and Wall, W. A. The use of domestic methylene blue in staining milk by the Breed method.....	307
Salmon, The sources and characteristics of the bacteria in decomposing....	85
Salt effects in bacterial growth. II. The growth of <i>Bact. coli</i> in relation to H-ion concentration.....	465
———, ———, ———, ———, ———, ———. III. Salt effects in relation to the lag period and velocity of growth.....	583
Salt effects in relation to the lag period and velocity of growth. Salt effects in bacterial growth, III.....	583
Saprophytic anaerobe, Quantitative determination of some of the biochemical changes produced by a.....	373
Sauerkraut, pink, The production of, by yeasts.....	257
Sherman, James M. and Holm, George E. Salt effects in bacterial growth. II. The growth of <i>Bact. coli</i> in relation to H-ion concentration.....	465
Sherman, J. M., Holm, G. E. and Albus, W. R. Salt effects in bacterial growth. III. Salt effects in relation to the lag period and velocity of growth.....	583
Single cells, Method for the isolation of bacteria in pure culture from, and procedure for the direct tracing of bacterial growth on a solid medium..	537
Skinner, W. W. and Koser, S. A. Viability of the colon-typhoid group in carbonated water and carbonated beverages.....	111
Society, Our.....	149
Soil, A method for counting the number of fungi in the.....	339
——, sulfur in the, Microorganisms concerned in the oxidation of. I. Introductory.....	231
——, ———, ———, ———, ———, ———, ———, ———, ———, ———. II. <i>Thiobacillus thiooxidans</i> , a new sulfur-oxidizing organism isolated from the soil.....	239





Wilson, G. S. The proportion of viable bacteria in young cultures with especial reference to the technique employed in counting.....	405
Yeasts, The production of pink sauerkraut by.....	257
Young cultures, The proportion of viable bacteria in, with especial reference to the technique employed in counting.....	405



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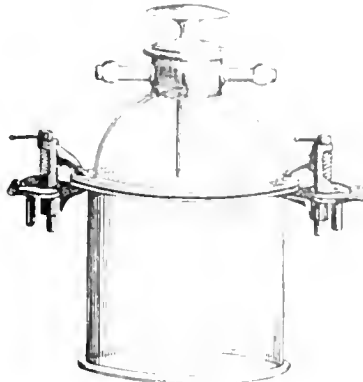
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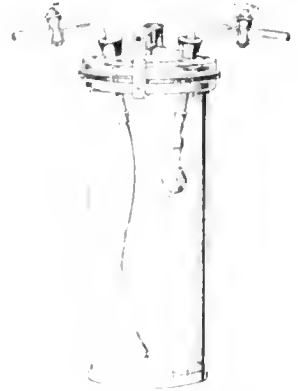
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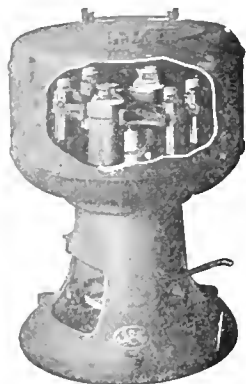
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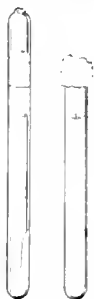
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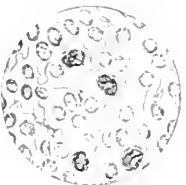
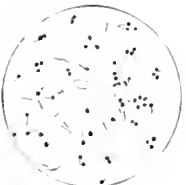
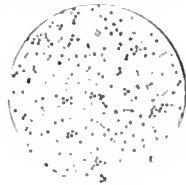
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## Contents

SEPTEMBER, 1922—Vol. VII—No. 5

- S. HENRY AYERS AND COURTLAND S. MUDGE. The Relation of Vitamines to the Growth of a Streptococcus.
- JAMES M. SHERMAN AND GEORGE E. HOLM. Salt Effects in Bacterial Growth. II. The Growth on *Bact. coli* in Relation to H-Ion Concentration.
- IVAN E. WALLIN. A Note on the Morphology of Bacteria Symbiotic in the Tissues of Higher Organisms.
- WILBERT C. DAVISON. Observations on the Properties of Bacteriolyants (D'Herelle's Phenomenon, Bacteriophage, Bacteriolytic Agent, etc.). Part I.
- WILBERT C. DAVISON. Observations on the Nature of Bacteriolyants (D'Herelle's Phenomenon, Bacteriophage, Bacteriolytic Agent, etc.). Part II.
- GEORGE F. REDDISH AND LEO F. RETTGER. *Clostridium putrificum* (*B. putrificus* Bienstock), a Distinct Species.
- J. HOWARD BROWN AND PAUL E. HOWE. Transparent Milk as a Bacteriological Medium.
- G. J. HUCKER AND W. A. WALL. The Use of Agar Slants in Detecting Ammonia Production and its Relation to the Reduction of Nitrates.
- H. J. CONN, K. N. ATKINS, H. J. BROWN, F. EBERSON, G. E. HARMON, G. J. HUCKER, F. W. TANNER AND S. A. WAKSMAN. Methods of Pure Culture Study. Report of Committee on Bacteriological Technic.
- H. J. CONN. An Investigation of American Gentian Violets. Report of Committee on Bacteriological Technic.

JULY, 1922—Vol. VII—No. 4

- L. D. BUSHNELL. Quantitative Determinations of Some of the Biochemical Changes Produced by a Saprophytic Anaerobe.
- G. S. WILSON. The Proportion of Viable Bacteria in Young Cultures with Especial Reference to the Technique Employed in Counting.
- H. J. CONN. A Method of Detecting Rennet Production by Bacteria.

MAY, 1922—VOL. VII—No. 3

- J. HOWARD MUELLER. Studies on Cultural Requirements of Bacteria. I.
- J. HOWARD MUELLER. Studies on Cultural Requirements of Bacteria. II.
- SELMAN A. WAKSMAN. A Method for Counting the Number of Fungi in the Soil.
- LETHE E. MORRISON AND FRED W. TANNER. Studies on Thermophilic Bacteria. I. Aerobic Thermophilic Bacteria from Water.
- ROBERT G. GREEN. An Apparatus for the Rapid Measurement of Surface Tension.

MARCH, 1922—Vol. VII—No. 2

- F. C. HARRISON. Our Society.
- VICTOR BURKE. Notes on the Gram Stain with Description of a New Method.
- BARNETT COHEN. Disinfection Studies. The Effects of Temperature and Hydrogen Ion Concentration upon the Viability of *Bact. coli* and *Bact. typhosum* in Water.
- SELMAN A. WAKSMAN. Microorganisms Concerned in the Oxidation of Sulfur in the Soil. I. Introductory.
- SELMAN A. WAKSMAN AND J. S. JOFFE. Microorganisms Concerned in the Oxidation of Sulfur in the Soil. II. *Thiobacillus Thiooxidans*, a New Sulfur-oxidizing Organism Isolated from the Soil.
- E. B. FRED AND W. H. PETERSON. The Production of Pink Sauerkraut by Yeasts.
- CONSTANTINO GORINI. Studies on the Biology of Lactic Acid Bacteria: A Summary of Personal Investigations.
- L. D. BUSHNELL. A Method for the Cultivation of Anaerobes.
- L. D. BUSHNELL. Influence of Vacuum upon Growth of Some Aerobic Spore-Bearing Bacteria.
- H. R. BAKER. Substitution of Brom-Thymol-Blue for Litmus in Routine Laboratory Work.
- W. A. WALL AND A. H. ROBERTSON. The Use of Domestic Methylene Blue in Staining Milk by the Breed Method.

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