TECHNICAL BULLETIN NO. 93 MARCH 1923

New York Agricultural Experiment Station

GENEVA, N. Y.

METHODS OF GRAM STAINING

G. J. HUCKER AND H. J. CONN

PUBLISHED BY THE STATION

BOARD OF CONTROL

GOVERNOR ALFRED E. SMITH, Albany
COMMISSIONER BERNE A. PYRKE, Albany.
IRVING ROUSE, Rochester.
FRANK M. BRADLEY, Barker.
MARTIN H. SMITH, Geneva.
CHARLES R. MELLEN, Geneva.
JOHN B. MULFORD, Lodi.
C. FRED BOSHART, Lowville.
PETER G. TEN EYCK, Albany.

OFFICERS OF THE BOARD

CHARLES R. MELLEN, *President.*
EDWARD J. COOK, *Secretary and Treasurer.*

STATION STAFF

ROSCOE W. THATCHER, D. Agr., *Director.*

†ALBERT R. MANN, A. M., *Agricultural Economist.*
GEORGE W. CHURCHILL, *Agriculturist.*
REGINALD C. COLLISON, M. S., *Chief in Research (Agronomy).*
†T. LYTTLETON LYON, Ph.D., *Chemist (Agronomy).*
JAMES E. MENSCHING, M.S., *Associate in Research (Agronomy).*
JAMES D. HARLAN, B.S., *Assistant in Research (Agronomy).*
WILLIAM P. WHEELER, *Associate in Research (Animal Industry).*
ROBERT S. BREED, Ph.D., *Chief in Research (Bacteriology).*
HAROLD J. CONN, Ph.D., *Chief in Research (Soil Bacteriology).*
†WILLIAM A. STOCKING, jr., M.S.A., *Bacteriologist.*
GEORGE J. HUCKER, M.A., *Associate in Research (Bacteriology).*
ARCHIE H. ROBERTSON, B.S., *Assistant in Research (Bacteriology).*
FRED C. STEWART, M.S., *Chief in Research (Botany).*
†DONALD REDDICK, Ph.D., *Botanist.*
†LEWIS KNUDSON, Ph.D., *Plant Physiologist.*
WALTER O. GLOYER, M.A., *Associate in Research (Botany).*
W. HOWARD RANKIN, Ph. D., *Associate in Research (Plant Pathology).*
EDWARD E. CLAYTON, Ph.D. (Riverhead). *Plant Pathologist.*
MANCEL T. MUNN, M.S., *Associate Botanist.*
ELIZABETH F. HOPKINS, A.B., *Assistant Botanist.*
LUCIUS L. VAN SLYKE, Ph.D., *Chief in Research (Chemistry).*
DWIGHT C. CARPENTER, Ph.D., *Associate in Research (Dairy Chemistry).*
LEON R. STREETER, B.S., *Assistant in Research (Insecticides and Fungicides).*
RUDOLPH J. ANDERSON, Ph.D., *Chief in Research (Biochemistry).*
†LEONARD A. MAYNARD, Ph.D., *Biochemist.*
MILLARD G. MOORE, B.S., *Assistant in Research (Biochemistry).*
ARTHUR W. CLARK, B.S., *Associate Chemist.*
MORGAN P. SWEENEY, A.M.,
WILLIAM F. WALSH, B.S., *Assistant Chemists.*
HAROLD G. BEATTIE, B.S.,
WALTER F. MORTON, B.S., *Assistant Chemists.*
ARTHUR C. DAHLBERG, M.S., *Associate in Research (Dairying).*
JAMES D. LUCKETT, M.S.A., *Editor and Librarian.*
KATHERINE S. OAKS, B.A., B.L.S., *Assistant Editor and Librarian.*
PERCIVAL J. PARROTT, M.A., *Chief in Research (Entomology).*
†GLENN W. HERRICK, B.S.A., *Entomologist.*
HUGH GLASGOW, Ph.D.,
FRED Z. HARTZELL, M.A., (Fredonia), *Associates in Research (Entomology)*
HUGH C. HUCKETT, M.S. (Riverhead), *Associate Entomologist.*
GUY F. MCLEOD, B.S.,
S. WILLARD HARMAN, B.S., *Assistants in Research (Entomology).*
ULYSSES P. HEDRICK, Sc.D., *Vice-Director; Chief in Research (Horticulture).*
†ROLLINS A. EMERSON, Sc.D., *Geneticist.*
†WILLIAM H. CHANDLER, Ph.D., *Pomologist.*
FRED E. GLADWIN, B.S., (Fredonia)
ORRIN M. TAYLOR,
GEORGE H. HOWE, B.S.A.,
RICHARD WELLINGTON, M.S., *Associates in Research (Horticulture).*
FRANK H. HALL, B.S., *Associate in Research (Vegetable Gardening and Canning Crops).*
HAROLD B. TUKEY, M.S.,
GEORGE L. SLATE, B.S.,
ALWYN BERGER, Ph.D. *Assistants in Research (Horticulture).*
JAMES S. LAWSON, Phm.B., *Museum Preparator.*
JESSIE A. SPERRY, *Director's Secretary*
FRANK E. NEWTON,
WILLARD F. PATCHIN,
LENA G. CURTIS,
MAUDE L. HOGAN,
K. LORAINE HORTON,
LUCY I. CLEARY, *Clerks and Stenographers.*
ELIZABETH JONES, *Computor and Mailing Clerk.*

Address all correspondence, not to individual members of the staff, but to the NEW YORK AGRICULTURAL EXPERIMENT STATION, GENEVA, N. Y.

The bulletins published by the Station will be sent free to any farmer applying for them.

†Members of the faculty of the New York State College of Agriculture affiliated with this Station.

TECHNICAL BULLETIN No. 93

METHODS OF GRAM STAINING

G. J. HUCKER AND H. J. CONN

SUMMARY

An historical discussion of the Gram method for staining bacteria is given, showing that the literature abounds in different technics all given this same designation. Nineteen of the most characteristic of these technics were selected for investigation.

It was found that results with any particular organism may vary considerably, according to the technic used, and that some of the procedures investigated are apparently more constant than others.

Finally, from various procedures, two particular stain formulæ were selected as giving the most constant results in the authors' hands. With them further tests were made concerning the different steps necessary in carrying out the Gram procedure.

As a result of the investigation no one particular technic is recommended. It is pointed out particularly that the Gram stain is a variable reaction even under the most carefully controlled conditions. No worker should pronounce any particular organism either positive or negative to the Gram stain after a single observation. It is recommended that, in order to determine the tendency of an organism with regard to the Gram stain, more than one staining procedure be used, and that preparations of the culture be prepared at various stages of growth from 12 hours to several days in age.

INTRODUCTION

About forty years ago, Gram (1884)[1] noted that certain bacteria in the presence of the pararosanilin dyes and iodine formed compounds insoluble in various solvents and advocated a staining method based on these phenomena for the demonstration of various organisms isolated from tubercle lesions, pneumonia, and other diseases which were under investigation at that period. In using this staining procedure, it was soon noted by others that only certain types of organisms had the power to produce the compounds that were insoluble in alcohol and these types became known as the "Gram-positive" types in contrast to the large number of "Gram-negative"

[1]See Bibliography, page 34.

organisms which failed to retain the violet stain in the presence of alcohol. In later years the method has been universally adopted as a procedure with diagnostic significance in many cases.

PREVIOUS STUDIES

The original method as advocated by Gram required the use of Ehrlich's anilin gentian violet solution which was prepared as follows:

Gentian violet	1 part
Alcohol	15 parts
Anilin	4 parts
Water	80 parts

Ehrlich had previously used this formula as a general bacterial stain but did not treat his preparations with iodine or with a solvent. The above solution, according to the directions given by Gram, was kept in contact with the preparation for one to three minutes, and then Lugol's iodine solution (iodine 1 part, potassium iodide 2 parts, distilled water 300 parts) was applied for a similar length of time. Lugol's iodine solution had been used previous to this time by bacteriologists and botanists as a general stain. The stained preparations were then decolorized with absolute alcohol for 30 seconds to remove the stain from those organisms which had not formed the above-mentioned insoluble compounds. In some cases clove oil was substituted for the alcohol in the final stages of the differentiation and especially was this true when organisms were studied in the tissues. In staining sectioned preparations the method was somewhat modified and various other stain solutions were used, such as anilin, fuchsin, etc.; and also various modifications of the iodine were tried but with no apparent success.

Since Gram's original publication, this technic has been modified and remodified an almost countless number of times. A search thru the literature reveals a surprising number of different procedures, all designated as the Gram method. To discuss all these modifications would require so much space that the best way to present them seems to be in tabular form, accordingly all of the distinct modifications which the writers have been able to find are listed in Table 1. Only a few of the most important of these need be given special mention.

One of the most frequently mentioned of these modifications is that of Weigert (1887), who published a technic which had proved successful in his laboratory for staining organisms in tissues. The

principal modification of this method was the use of a mixture of anilin oil (2 parts) and xylol (1 part) for decolorizing, instead of alcohol which had been commonly used by earlier workers.

Ethyl alcohol has not always been used alone as a decolorizer as Kisskalt (1901) found methyl alcohol to give more constant results. This observer noted that the molecular weight of the alcohol might have considerable effect upon the results. As a result of several tests, he stated that where gentian violet was used as a stain it could be extracted from the preparation at a rate inversely proportional to the molecular weight of the monovalent alcohols. Methyl alcohol would extract the stain the most vigorously, while ethyl, propyl, butyl, and amyl alcohol decolorized in proportion to their relative molecular weights. From this it was evident that organisms giving a positive reaction when butyl or amyl alcohol were used might not retain the violet stain if other alcohols were substituted.

Nicolle (1895) in differentiating organisms with the Gram technic devised several satisfactory modifications. In lieu of the anilin gentian violet he used:

Gentian violet (saturated solution in 95 per cent alcohol) 10 cc.
Phenol (1 per cent in water) ..... 100 cc.

This solution was found to give as satisfactory, if not better, results than Ehrlich's solution and was much better for use in staining sectioned preparations. Nicolle, whose primary interest was in staining organisms in tissues, modified the decolorizing solutions by the addition of eosin to the alcohol and also in some cases to the iodine mixture. A mixture of alcohol and acetone was also used in his laboratory with satisfactory results.

Claudius (1897) suggested a modification to be used in staining organisms in tissues which consisted in staining with a 1 per cent solution of methyl violet 6B, washing, applying a one-half saturated solution of picric acid, drying, and differentiating in chloroform or clove oil. No alcohol was used for differentiation nor was anilin oil used in the stain mixtures.

Of the recent modifications of the Gram technic, that of Stirling[2] and Atkins (1920) have received the most attention in the United States. The former method requires a more concentrated stain solution (5 per cent) and shorter staining periods (30 seconds). This

[2]The authors have been unable to find the original paper in which the Stirling method was described and are of the opinion that it has never actually been published under Stirling's name.

TABLE 1.—VARIOUS MODIFICATIONS OF THE GRAM STAIN.

AUTHOR	VIOLET STAIN	IODINE SOLUTION	DECOLORIZING AGENT	COUNTER-STAIN
Stirling	**5 grams of stain ground in mortar with 10 cc. of 95 per cent alcohol. Filter and add 2 cc. of anilin oil and 88 cc. of water (1 minute).**	**Lugol's (1 minute)**	**95 per cent alcohol (2 minutes)**	———
Löffler, 1884	10 cc. carbol methyl violet 6B and 1 cc. alcoholic methylene blue solution. Wash.	Lugol's (2 minutes)	5 per cent aqueous solution of nitric acid (1 minute), or 3 per cent HCl in alcohol (10 seconds), or 30 per cent acetone in alcohol	Dilute fuchsin solution
Günther, 1887	———	———	HCl Alcohol (10 seconds)	———
Unna, 1888	———	5 per cent KI plus H_2O_2	———	———
Weigert; modified by Kühne, 1888	Lithia carmine solution (½ hour). Differentiate in alcohol or HCl alcohol solution. Wash, stain with crystal violet (concentrated aqueous with a drop of HCl) for 5 to 15 minutes, wash, and dry with blotting paper.	Lugol's (1 to 2 minutes), dry.	Anilin oil	———
Novy, 1899	Anilin gentian violet (10 to 15 minutes), wash.	Lugol's (3 to 5 minutes)	Absolute alcohol	Dilute eosine (½ minute), dehydrate 1 to 2 minutes, oil of cloves
Kütscher, 1894	Anilin water gentian violet, alcohol, and 5 per cent phenol (equal parts), 10 to 15 minutes.	———	———	———

Nicolle, Method I, 1895	**10 cc. alcoholic gentian violet and 100 cc. 1 per cent phenol (1 to 5 minutes).**	**Lugol's (4 to 6 seconds)**	**3 parts absolute alcohol and 1 part acetone.**	———
Nicolle, Method II, 1895	Alcoholic carmin solution (5 parts Orth's carmin and 1 part 95 per cent alcohol). Stain with gentian violet as in Method I.	Lugol's (4 to 6 seconds)	30 per cent by volume of acetone in absolute alcohol	95 per cent alcohol and picric acid until yellow-green (1 to 5 seconds), carbol-fuchsin (20 seconds)
Claudius, 1897	1 per cent solution of methyl violet (1 minute), wash, dry, wash in picric acid solution (1 minute), wash and dry.	———	Chloroform	———
Jordan, 1908	**75 parts of anilin water (anilin 2 cc., water 98 cc.) and 25 parts saturated alcoholic solution (2 minutes)**	**Lugol's (1½ minutes)**	**95 per cent alcohol for at least 5 minutes**	———
Stephan, 1909	10 cc. saturated alcoholic solution of methyl violet 6B and 40 cc. 2 per cent phenol (10 minutes to 1 hour)	10 per cent solution of potassium ferric cyanide (1 part) and 5 per cent iodine solution (2 parts) for 10 minutes, wash.	Absolute alcohol	———
Eisenberg, 1910	1 per cent solution Victoria Blue (3 to 5 minutes), wash.	Lugol's (1 to 2 minutes)	Nicolle's acetone alcohol solution until no more color is removed, wash.	Carbol-fuchsin
Buchanan, 1911	**6 cc. of saturated alcoholic solution of stain and 50 cc. anilin water (1 to 2 minutes)**	**Lugol's (1 to 2 minutes)**	**95 per cent alcohol until no more color is removed**	———
Stitt, 1911	**25 cc. of saturated alcoholic solution and 75 cc. of formalin (1 minute)**	**Lugol's (1 minute)**	**Alcohol until no more color is removed**	———
Jensen, 1912	0.5 per cent solution of methyl violet	Solution of 1 gram iodine, 2 grams KI, and 100 cc. H_2O.	———	———

TABLE 1. — CONTINUED

AUTHOR	VIOLET STAIN	IODINE SOLUTION	DECOLORIZING AGENT	COUNTER-STAIN
Moore, 1912	**Mixture of 5 per cent solution of phenol and saturated alcoholic solution of stain 1 to 20 parts (5 to 7 minutes)**	———	———	———
Mallory and Wright, 1913	Lithia-carmine solution (2 to 5 minutes), dehydrate with alcohol. Anilin methyl violet for 5 minutes.	———	Anilin oil	———
Mallory and Wright, 1913	Anilin methyl violet (5 to 20 minutes)	Lugol's	Absolute alcohol	———
Eyre, 1915	Solution of 3 drops anilin water and 15 drops alcoholic solution of gentian violet (30 minutes)	———	Anilin oil and nitric acid, wash and treat with equal parts anilin oil and xylol.	———
Stovall, 1916	**Solution of anilin oil, 28 cc.; saturated alcoholic stain,8 cc.; 95 per cent alcohol, 100 cc.; normal HCl, 5 cc.; and water, 1000 cc. (1 minute)**	**Lugol's (1 minute)**	**95 per cent alcohol until no more color can be removed**	
Leidy, 1919	———	Iodine 1 gram, ferrous or arsenic iodide 2 grams, and H_2O, 300 cc.	———	———
Lyon, 1920	Anilin gentian violet	Lugol's	Acetone	———
Atkins, 1920	**1 part of saturated alcoholic solution of stain and 3 parts 0.1 per cent solution of anilin sulfate (1 minute)**	**2 grams of iodine, 10 cc. normal NaOH, and 90 cc. water (1 minute)**	**95 per cent alcohol (1 minute)**	———
Hucker, I, 1921	Anilin gentian violet	Lugol's	Solution of anilin oil 2 parts, xylol 3 parts, and alcohol 95 parts.	Bismarck brown

Burke, 1921	1 per cent aqueous solution, stain for 2 to 3 minutes then add 3 to 8 drops 5 per cent solution sodium carbonate.	Lugol's (1 minute)	Acetone or solution of ether (1 part) and acetone (3 parts), decolorize until no more color can be removed.	———
Orla-Jensen, II, 1921	0.5 per cent aqueous solution of stain (1 minute)	Lugol's(1 minute)	95 per cent alcohol until no more color can be removed	———
Hucker, II, 1922	1 part of saturated alcoholic solution of stain and 4 parts of 1 per cent aqueous solution of ammonium oxalate (1 minute)	Lugol's (1 minute)	95 per cent alcohol (1 minute)	———
Tunnicliff, 1922	Carbol gentian violet	Lugol's	Wash, do not decolorize.	———
Hoffman*	Grind 0.5 gram of stain with 10 cc. of 95 per cent alcohol in mortar, filter, add 90 cc. of 2.5 per cent solution phenol to 10 cc. of above filtrate (20 minutes).	Lugol's (20 seconds)	95 per cent alcohol until no more color can be removed	———
Gradwohl*	5 grams of stain, 10 cc. of 95 per cent alcohol, 2 cc. of anilin oil, and 8 cc. of water (25 seconds).	———	95 per cent alcohol until no further color can be removed	———
Murray, Purwin and McNutt.†	A solution of 28 cc. of anilin oil, 5 grams of stain, 100 cc. of 95 per cent alcohol, and 1000 cc. of water is applied for 1 minute. Wash.	Lugol's (30 seconds)	95 per cent alcohol (1 minute)	———
Gorham*	0.5 gram stain, 1.5 cc. of 95 per cent alcohol, and 2.2 cc. of anilin oil ground in mortar. Stand for 24 hours, add 8.8 cc. distilled H_2O, filter (30 seconds).	Lugol's (30 seconds)	95 per cent alcohol until no more color is removed	———

*These methods were used by the investigators named above in the investigation of American gentian violets made by the Committee on Bacteriological Technic (1922). The original authors of the methods have not yet been determined.

†This method is denoted in the later tables by the abreviation M. P. & McN.

TABLE 1 — CONCLUDED.

AUTHOR	VIOLET STAIN	IODINE SOLUTION	DECOLORIZING AGENT	COUNTER-STAIN
Harrison*	0.5 gram of stain added to 20 cc. of solution of equal parts of saturated aqueous anilin water, absolute alcohol, and 5 per cent phenol (30 seconds)	Lugol's (1 minute)	95 per cent alcohol (1 minute)	————
Hachtel*	1 cc. of a solution containing 0.4 gram of stain, 1.8 cc. of anilin oil, and 6.6 cc. alcohol is added to 9 cc. of solution of 0.4 gram stain in 20 cc. of water. Filter (3 minutes).	Lugol's (2 minutes)	25 per cent alcohol until no more color is removed	————
Modification A**	0.5 per cent alcoholic solution	Lugol's	95 per cent alcohol	Safranin
Modification B**	Saturated alcoholic solution of stain and 1 per cent N/10 NaOH	Lugol's	95 per cent alcohol	Safranin

*These methods were used by the investigators named above in the investigation of American gentian violets made by the Committee on Bacteriological Technic (1922). The original authors of the methods have not yet been determined.
**These last two modifications were used in the present work, for purposes of comparison only.

method has proved satisfactory, but due to the presence of the anilin oil the stain is not stable and must be prepared before each using. The high concentration of stain also favors the deposit of a large amount of precipitate on the slide. The Atkins modification attempts to do away with the objection to the former method and substitutes anilin sulfate for the anilin oil as a mordant. A modified iodine solution (iodine, NaOH, and H_2O) is used. This liberates the anilin from the anilin salt when the iodine solution is applied to the slide. The latter method has several advantages over the more commonly used Stirling method, as the solutions are stable and the resulting preparations are clear with the organisms stained very distinctly.

Various other minor modifications have appeared, as noted in Table 1, but the variations have usually been only in the concentration of the different solutions or special manipulation in the routine procedure of the staining. One very recent modification which is still more different is that of Scales (1922) who has employed an entirely new dye, cotton blue C_4B (Poirrier's Blue), which he finds to work very similarly and without the need of treatment with iodine after staining. The advantage of eliminating one step in the procedure is obvious, but the method of Scales has appeared so recently that it could not be included in the present investigation.

DYES USED IN GRAM STAINING

The dyes used by the different workers in the initial staining had been either methyl violet or gentian violet. Both of these names, however, are used rather indefinitely to refer to certain mixtures of pararosanilins. The important compounds in these mixtures are:

Tetramethyl pararosanilin, *viz.*, $(CH_3)_2N \cdot C_6H_4 \cdot C \begin{cases} C_6H_4 \cdot NH_2 \\ C_6H_4 = N(CH_3)_2Cl \end{cases}$

Pentamethyl pararosanilin, *viz.*, $(CH_3)_2N \cdot C_6H_4 \cdot C \begin{cases} C_6H_4 \cdot NH(CH_3) \\ C_6H_4 = N(CH_3)_2\,Cl \end{cases}$

Hexamethyl pararosanilin, *viz.*, $(CH_3)_2N \cdot C_6H_4 \cdot C \begin{cases} C_6H_4 \cdot N(CH_3)_2 \\ C_6H_4 = N(CH_3)_2\,Cl \end{cases}$

Gentian violet is a name that was given by Grübler to a mixture of these three compounds, probably with certain other pararosanilins; but there seems to be no definite agreement as to just what this

mixture contained, so that at present different concerns are selling different mixtures under the name of gentian violet. Methyl violet, however, is more definitely understood. Various grades are sold under the trade desgination of R, 2R, 3R, B, 2B, 6B, etc., these designations indicating not the chemical composition but the shade of the dye. The more B's attached to the name the bluer its color, or the more R's the redder. Of the three compounds just listed those of lower methylation are the redder in shade and those of higher methylation the bluer; hence, in general, the number of B's in the trade designation indicates the proportion of the more highly methylated compounds that are present in the mixture. Methyl violet 6B is supposed to contain a compound in which one of the methyl groups has been replaced by a benzyl group and is sometimes known as benzyl violet. The dye known as crystal violet is, or should be hexamethyl pararosanilin chloride alone.[3]

In the comparative tests given below, representatives of different violet pararosanilines have been used, in order to avoid, if possible, erroneous conclusions due to stains that are not well adapted to the Gram procedure.

COMPARISON OF VARIOUS METHODS OF GRAM STAINING

The principal factor involved in the technic of Gram staining is the use of some mordant in the violet stain solution which will insure constant results even when vigorously decolorized. This particular point has stimulated laboratory workers to try other mordants in addition to those mentioned above, such as formalin, sodium hydroxide, etc.; while some laboratories, especially those of Europe, report that an aqueous solution of methyl violet with no mordant is satisfactory. In nearly all instances the mordant has been suitable but the staining solutions have been very unstable, as for example in the case of the anilin-oil-gentian violet. Various methods have been employed to obviate this objection to the use of anilin oil, the most common being the addition of phenol or its substitution for anilin oil.

There has been a general recognition of the fact that none of these mordants have given an absolutely clear-cut distinction between the organisms that decolorize and those that retain the stain. Hence, the time of decolorization has been varied by different bacteriologists in an

[3]For a more detailed account of the stains involved consult Mann (1902). See also Committee on Bacteriological Technic (1922).

effort to secure more constant results. In a survey of approximately 50 different methods, it was noted that the time of decolorization varied from 30 seconds to several minutes. Similar variations in the concentration of the dye have been noted, undoubtedly in the hope that the proper adjustment of dye strength would give a technic that would allow a constant differentiation between the negative and positive organisms.

After a careful comparison of the various published methods, including not only those in Table 1, but certain others differing so slightly from some of these that they have not been included in the table, it became evident that about 20 basic procedures could be selected of which the others are merely modifications. Nineteen of these more important methods were selected for use in the present investigation. These 19 methods are printed in Table 1 in bold-faced type.

One of these 19 methods, namely that published by the senior author (Hucker, 1922), deserves special mention. This technic calls for ammonium oxalate as a mordant, altho its use for this purpose has never been suggested elsewhere. The technic was first used by the writers in an entirely accidental way, due to a mistaken impression that one of the other authors whose work was being followed had used this reagent. Before the mistake was discovered, this new technic had been compared with a long series of other procedures and had been found to give such surprisingly good results in comparison with the others as to recommend it for general use. Its chief advantage is that the solution is practically stable, and yet its use calls for no modification of the usual Gram technic further than the addition of this chemical to the solution of dye.

METHODS USED IN TESTING AND SCORING VARIOUS PROCEDURES

As stated above, in order to determine a procedure that would be satisfactory with dyes from various sources, two or three different dyes of the violet pararosanilin group were selected. The four stains used in the work reported in Tables 2 and 3 were as follows: A gentian violet and a crystal violet purchased from one of the domestic firms specializing in biological stains, a methyl violet 6B purchased from a domestic dealer in textile dyes which had been found rather unsatisfactory for the Gram stain in a series of preliminary investigations, and a gentian violet of Grübler origin imported before 1914. In the work reported in the later tables, only

one dye was used, a crystal violet purchased from one of the domestic basic dye manufacturers (Dupont) which had proved in an investigation of the Committee on Bacteriological Technic to be one of the best dyes for the Gram stain.

In the work reported in Table 2, three organisms were used, *viz.*, *Bacillus cereus*, as a representative of the strongly Gram-positive group; a short rod of the fluorescent group of bacteria, as a representative of the distinctly Gram-negative types; and a micrococcus isolated from cheese which had proved to be variable to the Gram stain with a tendency to be positive more often than negative. For the purposes of the present investigation this coccus was assumed to be Gram-positive, and a technic was not considered perfect unless both the coccus and *B. cereus* retained the stain, while the short rod was decolorized. Twenty-four-hour cultures of these three organisms were used and preparations of all three were made on each of the slides to be examined. The slides were all stained by one of the writers, following as nearly as possible the times for the different procedures as indicated by the authors of the different methods; but in this early work, before it was realized how great the results might vary with the timing of the various steps, there was probably less constancy in this respect than in the later work.

After staining, the slides were examined by three different observers to eliminate the personal equation in interpreting results. In tabulating results a system of scoring was adopted which was more or less arbitrary and has only comparative value. From a score of ten a large deduction was made if a precipitate was present which covered the organisms and obscured the results; a moderate deduction if *B. cereus* appeared negative or the short rod positive; and a smaller deduction if the coccus showed the negative reaction. The individual scores for each slide were averaged and also an average made, on the basis of 100, for the four dyes used and listed as the "total average" in the tables given below.

A similar score was computed for the keeping quality of the various dye solutions with the final observation taken at the end of three months. The points considered in this scoring were: (a) Length of time before the stain decomposed, (b) staining quality after three months, and (c) nature of decomposed solution.

RESULTS OF COMPARATIVE TEST OF METHODS

Of the 19 methods used in the preliminary tests only 4 were found

to be so unsatisfactory that they were eliminated from further testing. (See Table 2.) They were those of Gradwohl, Moore, Hoffman, and Stovall, which gave large amounts of precipitate that obscured the bacteria in many cases. Modification A, in which no mordant was used, also received a low score (50), but the preparations were brilliant and the organisms well defined, so that the results warranted retaining it for further testing.

As recorded in Table 2, the methods using anilin oil as a mordant received an average score, excluding the four methods which were discarded, of 82; while those which required phenol received an average score of only 72.

TABLE 2.—A COMPARISON OF VARIOUS METHODS OF MAKING THE GRAM STAIN, FIRST TEST.

Figures indicate the score of each preparation according to plan of grading described on page 14.

METHOD*	SCORES OF INDIVIDUAL PREPARATIONS — Stains used: gentian violet (Grübler)	gentian violet (domestic)	crystal violet (domestic)	methyl violet (domestic)	TOTAL AVERAGE SCORE	REMARKS
SIMPLE ANILIN OIL METHODS						
Stirling	8	5	9	9	77	
Jordan	7	5	10	9	77	
Buchanan	9	10	10	9	95	
Gradwohl	7	0	9	0	40	Heavy ppt.
Gorham	8	8	4	9	72	
Hachtel	4	6	8	5	77	
M. P. & McN.	8	9	10	10	92	
METHODS CALLING FOR PHENOL						
Nicolle I	9	10	2	5	65	
Moore	4	6	8	2	50	Heavy ppt.
Hoffman	7	0	5	0	30	Heavy ppt.
METHODS CALLING FOR NO MORDANT						
Jensen	10	10	4	2	65	
Burke	6	8	9	8	77	
Mod. A	5	5	5	5	50	
Mod. B	6	6	6	5	57	
SPECIAL METHODS						
Harrison†	5	7.5	9	9	76	
Stitt	4	7	7	6	60	
Stovall	4	10	4	2	50	Heavy ppt
Atkins	9	10	4	9	80	
Hucker II	9.5	10	10	7	90	

*The name or abbreviation in this column indicates the method so designated in Table 1.
†Harrison's method calls for both anilin and phenol.

In the second series of tests as shown in Table 3, the above procedure was followed with the exception that three new cultures (*B. diphtheriae*, *B. coli*, and *B. megatherium*), were used, as well as the three in the first series of tests. The preparations were all placed on the same slide and stained and scored as in the first tests.

The results really varied more than is indicated in the scores in the table and some additional observations should be noted. Method I of Nicolle, the only procedure in this series which requires phenol as a mordant, gave good results with both the domestic and foreign gentian violets, while the results with methyl and crystal violet were very unsatisfactory. The same condition held true in those cases where aqueous solutions were used with the addition of mordants. These facts indicate that phenol is not an efficient mor-

TABLE 3.—A COMPARISON OF VARIOUS METHODS OF MAKING THE GRAM STAIN, SECOND TEST.

Figures indicate score of each preparation according to the plan of grading described on page 14.

METHOD*	SCORES OF INDIVIDUAL PREPARATIONS — Stains used: gentian violet (Grübler)	gentian violet (domestic)	crystal violet (domestic)	methyl violet 6B (domestic)	TOTAL AVERAGE OF STAINING SCORE	AVERAGE SCORE OF KEEPING QUALITY	TOTAL AVERAGE SCORE
SIMPLE ANILIN OIL METHODS							
Stirling	8	8	8	8	80	50	65
Jordan	9	9.5	10	9	94	0	47
Buchanan	8	7.5	8	9	81	0	40
Gorham	5.5	8.5	10	5.5	72	100	85
Hachtel	4	6	8	5	77	80	77
M. P. & McN.	7	8	5.5	5	64	25	45
METHOD CALLING FOR PHENOL							
Nicolle I	9	10	2	5	65	80	72
METHODS CALLING FOR NO MORDANT							
Jensen	9.5	10	4	2	65	90	78
Burke	6	6	7	6	60	90	75
Mod. A	5	5	5	5	50	100	75
Mod. B	5	5	6	5	50	50	50
SPECIAL METHODS							
Harrison†	8	8	9	8	80	25	52
Stitt	0	5	5	0	20	90	55
Atkins	9.5	9	9.5	9.5	94	100	97
Hucker II	9.5	9.5	10	9.5	96	100	98

*The name or abbreviation in this column indicates the method so designated in Table 1.
†Harrison's method calls for both anilin and phenol.

dant, even when used with the gentian violet solutions. The method which requires an alcoholic solution only (Mod. A) is very unsatisfactory, altho the score given was 50. In this case only the Gram-negative organisms were properly stained which, according to the system of scoring, necessitated a 50 per cent score. Alcoholic solutions of anilin dyes are not efficient staining solutions and the above method is no exception.

From the standpoint of staining quality, the methods of Atkins and of Hucker were superior to the other methods tested. The organisms were evenly stained, no precipitate was formed, and both the anilin sulfate and ammonium oxalate have sufficient mordanting power to allow ample time for decolorizing. The methods of Jordan and Buchanan were equally satisfactory in some cases, but failed to give as clear preparations. This was due to the washings used between each step in the former procedures; while, in the methods of Jordan and Buchanan, the preparations were not washed. Altho the anilin-violet solutions give satisfactory results, they are not stable. The staining solutions of Jordan and Buchanan were both decomposed at the end of three weeks. When these methods are used in general routine work, fresh mixtures must be prepared at least once a week. These disadvantages are largely overcome when ammonium oxalate or anilin sulfate is used, as stains containing these mordants remain stable indefinitely. The authors have used stain solutions containing ammonium oxalate six months old with results apparently as satisfactory as with fresh solutions.

EFFECT OF LENGTH OF TIME OF DECOLORIZATION UPON THE GRAM STAIN

In order to test further the action of ammonium oxalate, anilin sulfate, and anilin oil as mordants, four preparations of the diphtheria organism were stained one minute with the solutions of Hucker, Atkins, Jordan, and Buchanan, respectively; treated one minute in iodine; decolorized for different lengths of time with ethyl alcohol; and then counter-stained 30 seconds with safranin. The results are shown in Table 4. The organisms when stained with the solution containing anilin oil as a mordant withstood the action of the alcohol for two minutes, after which the violet color was entirely gone. At the end of 30 minutes of decolorization many organisms were still found to retain the violet color, in the case of the organisms stained with a violet stain containing anilin sulfate; while the organisms

TABLE 4.—REACTION TO THE GRAM STAIN OF THE DIPHTHERIA ORGANISM AFTER VARIOUS PERIODS OF DECOLORIZATION WITH 95 PER CENT ALCOHOL.

METHOD*	LENGTH OF TIME DECOLORIZED					
	2 min.	10 min.	30 min.	1 hr.	2 hrs.	3 hrs.
Hucker II.....	+	+	+	±	±	±
Atkins.....	+	±	±	—	—	—
Jordan.....	+	—	—	—	—	—
Buchanan.....	+	—	—	—	—	—

*These methods were the same as those designated under the names of their respective authors in Table 1, except that the following uniform times for the various procedures were used: 1 minute in staining solution, 1 minute in iodine, and 30 seconds in counter-stain.

stained by the ammonium oxalate method still resisted the decolorizing action of the alcohol. Even at the end of three hours in the alcohol, many Gram-positive organisms were found in the preparations stained with the latter procedure.

These results indicate the relative action of the mordants in question rather than the optimum time to use for decolorization. They show that in the case of the first two methods (anilin sulfate and ammonium oxalate) there is such latitude in the time during which the bacteria retain the stain that a greater constancy of results is insured than when the anilin oil method is used.

In staining several cultures with various methods and varying the time of decolorization, it was noted in practically all cases that the effect of decolorizing is largely dependent upon the nature of the mordant used. However, as in the case in which *B. diphtheriae* was decolorized for various lengths of time, there was always found a narrow latitude of time, usually between one and two minutes, within which no apparent effect of the mordant could be noted upon the results. If, however, the variation in the time of decolorizing was allowed to extend beyond these limits of variation, noticeable effects upon the results were found. The range within which this variation of decolorizing time must be limited is dependent upon so many factors, *viz.*, strength of the alcohol, time of staining with the violet stain, nature of the mordant used, etc., that no general conclusions can be drawn. But it is evident that ammonium oxalate or anilin sulfate used as a mordant greatly increases this latitude of allowable variation.

Altho many laboratory workers feel that the time of decolorization in the Gram stain must be standardized, it is evident that the same results could be obtained by using a mordant in the violet stain which would allow ample variation in the decolorization time and then place a maximum and minimum time between which any possible variation might give acceptable results. Such a procedure would insure a greater constancy of results between different laboratories and would make the Gram stain a more valuable procedure in the hands of a beginner.

After some consideration of this point, it was decided in the following work to use a uniform time of decolorization. For this purpose 60 seconds was chosen. It was also decided that all the periods of staining and mordanting should be uniform, and the following procedure was always observed in the following work, regardless of the directions given by the author of each particular method used: 60 seconds in the violet stain, 60 seconds in iodine; 60 seconds decolorization; and 30 seconds in the counter-stain.

EFFECTS OF VARIOUS STRENGTHS OF ALCOHOL UPON THE RESULTS OF THE GRAM STAIN

Kisskalt (1901) and later Burke (1922) have shown that the strength of the alcohol plays an important part in the results of the Gram stain. Both of these observers found that the Gram-negative organisms gave up the violet stain much more slowly when diluted solutions of alcohol were used. The former (Kisskalt) found also that the power of the alcohol to remove the violet stain was in close relation to the molecular weight of the alcohol, while Burke noted that the amount of the dilution of the alcohol had a close relation to the decolorizing power, *viz.*, the greater the dilution of the alcohol the less active as a decolorizer. Burke also found that if the decolorizing alcohol was greatly diluted sufficient distinction could not be found between the time of decolorization of the Gram-negative and Gram-positive organisms, with the result that both would decolorize at practically the same rate.

In order to secure additional data on this particular point, preparations were made from 24 different organisms and stained, both with the ammonium oxalate formula (Hucker, 1922) and the anilin oil method of Buchanan (1911). The 24 organisms used in this test were carefully selected from a series of cocci and short rods obtained from various sources. These particular cultures were selected be-

cause they had been found to be extremely variable to the Gram stain in a series of preliminary tests; and, in order to make the present test as severe as possible, it was decided that the organisms should be as variable in their relation to the Gram stain as they possibly could be.

Slides bearing preparations of these 24 bacteria (12 on each slide) were stained by the two above methods and then decolorized one minute in different strengths of alcohol. It was considered that the best strength of alcohol would be the one giving the greatest constancy in results; hence three slides were decolorized in each strength of alcohol, and the results from each separate slide are tabulated separately.

The results are noted in Tables 5 and 6. With both the anilin oil and ammonium oxalate methods, it was found that the greater the dilution of the alcohol the more slowly the violet stain was removed from the preparations. In the case of 60 per cent alcohol used for five minutes, it can be seen from Table 5 that the Gram-negative and Gram-positive organisms each decolorized at about the same rate, so that many of the Gram-positive strains appeared to be negative in reaction.

It is evident that the strength of the alcohol plays an important part in the results of Gram staining, and any procedure, such as washing after the application of the iodine solution which might dilute the alcohol, must be avoided. If care is taken, however, and the slide well drained before the use of the alcohol and sufficient alcohol used, little trouble will be experienced on this particular point.

An interesting comparison between the use of 95 per cent and absolute alcohol can be noted also in the same tables. From the standpoint of constancy no apparent difference in the results as secured with either of these strengths would be noted; evidently 95 per cent alcohol is just as effective a decolorizing agent as absolute alcohol.

Both the anilin oil and ammonium oxalate methods gave similar results and it is to be expected that comparable results would be obtained regardless of the method used.

COMPARISON OF ALCOHOL (95 PER CENT) AND ACETONE AS DECOLORIZING AGENTS

Many laboratory workers prefer acetone to alcohol for use in decolorizing preparations stained with the Gram technic. Many of

the earlier workers reported good results with acetone and a few used it in combination with other reagents in decolorizing, *viz.*, anilin oil, xylol, etc.

From the above results it is evident that 95 per cent alcohol is adaptable for the Gram stain and gives as good results as absolute alcohol. As a comparison of acetone and alcohol,[4] the 24 above-mentioned cultures were stained with an anilin oil method (Buchanan) and with the ammonium oxalate method. The methods were like those in the work reported in Tables 5 and 6, except that in each case duplicate preparations were decolorized, one with alcohol and one with acetone. Table 7 shows the results in outline. It can be seen that in the case of the ammonium oxalate method, acetone did not give as constant results as were obtained with alcohol, but a larger number of organisms were decolorized with alcohol than with acetone. In the case of the anilin oil method, however, the acetone gave much more constant results than the alcohol, but in this case also the alcohol was found to have a more powerful effect in removing the violet stain from the organisms than did the acetone.

The length of time of decolorizing appeared to have no appreciable effect upon the action of the acetone, and it failed to remove the violet stain in some cases even when allowed to act for several minutes. From such results it is difficult to draw conclusions, as in some instances the acetone may cause an organism which naturally should be classed with the Gram-negative group to retain its violet stain, while in other cases alcohol might remove the stain from cultures which should be Gram-positive.

In order to secure additional data on this particular point and to compare the action of acetone with alcohol when used on a longer series of cultures, 135 cultures of cocci were selected and stained in duplicate by the same Gram technic, then one decolorized with alcohol, the other with acetone, in each case decolorizing until no more stain could be removed, usually not over one minute. In this case the alcohol acted as a less powerful destaining agent than the acetone. In 29 cases organisms appeared as Gram-negative when decolorized with acetone; while in a duplicate preparation decolorized with alcohol they failed to retain the violet stain, and in only 11 cases was the reverse true.

These results indicate that acetone is not as constant as alcohol as

[4]In all cases where alcohol is mentioned without the strength being given, the authors refer to 95 per cent alcohol.

TABLE 5.—REACTION TO THE GRAM STAIN OF VARIOUS CULTURES STAINED IN TRIPLICATE AND DECOLORIZED 1 MINUTE IN DIFFERENT STRENGTHS OF ALCOHOL, USING THE AMMONIUM OXALATE METHOD (HUCKER, 1922).*

DECOLORIZING AGENT	CULTURE NUMBER																							
	$M4_2$	D_1	G_2	D_6	X_4	D_8	$X1_1$	$W5_2$	$P31_1$	DS_1	116	213	40	148	154	37	90	91	195	149	201	382	155	97
100 per cent alcohol	±	+	—	±	—	+	+	+	+	+	+	—	±	+	+	+	+	+	+	—	+	±	+	±
	—	+	—	±	—	+	±	+	+	+	+	—	±	+	+	+	+	±	+	—	+	±	+	±
	—	+	—	—	—	+	+	+	+	+	+	—	±	+	+	+	+	+	±	—	+	±	+	+
95 per cent alcohol	±	+	—	—	—	+	+	+	+	+	+	—	±	±	+	+	±	+	+	—	±	±	±	+
	+	+	—	—	—	+	+	+	+	+	+	—	±	±	+	+	+	+	+	—	+	+	±	+
	+	+	—	—	—	+	+	+	+	±	+	—	±	±	+	+	+	+	+	—	±	+	±	+
85 per cent alcohol	—	+	—	±	—	+	+	+	+	±	+	—	±	—	±	+	±	—	—	±	±	+	—	+
	—	—	—	±	—	+	+	+	+	—	±	—	—	±	—	±	±	—	—	—	±	±	±	±
	—	—	—	—	—	+	+	+	+	+	+	—	±	+	+	+	±	—	—	—	±	+	±	+
70 per cent alcohol	—	—	—	±	—	+	+	+	+	±	±	—	+	+	+	+	+	+	—	—	±	+	±	+
	—	—	—	+	—	+	+	+	+	+	±	—	—	—	—	—	—	—	—	—	+	+	—	—
	+	±	—	+	—	+	+	+	+	—	±	—	+	+	—	±	±	—	—	—	+	+	—	±

*This method was used with 1 minute staining, 1 minute in iodine, and 30 seconds in safranin as a counter-stain.

TABLE 6.—REACTION TO THE GRAM STAIN OF VARIOUS CULTURES STAINED IN TRIPLICATE AND DECOLORIZED 1 MINUTE IN DIFFERENT STRENGTHS OF ALCOHOL, USING THE ANILIN OIL FORMULA OF BUCHANAN.*

DECOLORIZING AGENT	CULTURE NUMBER																								
	$M4_2$	D_1	G_2	D_6	X_4	D_8	$X1_4$	$W5_2$	$P31_1$	DS_1	116	213	40	148	154	37	90	91	195	149	201	382	155	97
100 per cent alcohol	—	+	—	—	—	+	—	+	+	—	±	—	+	±	±	—	—	+	+	+	+	+	+	+
	+	+	—	±	—	+	±	±	+	—	+	±	—	±	±	±	±	+	+	+	±	±	+	+
	±	+	—	—	—	+	±	±	+	±	±	—	—	+	+	±	±	—	+	+	±	+	+	+
95 per cent alcohol	+	+	—	—	—	+	±	—	+	±	+	—	—	+	+	+	+	+	+	+	+	+	+	+
	—	+	—	—	—	±	—	—	+	—	—	—	—	±	±	—	—	—	—	±	±	—	—	+
	—	+	—	0 †	—	+	—	—	±	—	—	—	—	+	—	—	—	+	+	+	+	+	+	+
85 per cent alcohol	—	±	—	—	—	+	—	±	±	—	—	—	±	—	—	—	—	—	—	—	—	+	—	+
	—	—	—	—	—	±	—	±	±	—	—	—	—	—	—	—	—	—	—	—	±	+	—	±
	—	—	—	0 †	—	±	±	+	±	—	—	—	±	—	—	—	—	—	±	—	—	+	—	±
70 per cent alcohol	+	±	±	±	±	+	±	±	—	—	±	—	±	—	—	—	—	—	+	±	—	+	±	±
	—	±	—	—	—	+	+	+	±	±	±	—	±	—	—	—	—	—	—	—	±	+	—	—
	—	±	—	—	—	±	—	+	+	±	+	—	±	—	—	—	—	—	—	—	—	+	—	±
60 per cent alcohol	+	+	+	+	+	+	+	+	+	+	+	+	±	+	+	+	+	+	±	+	+	+	+	±
	+	+	+	+	+	+	+	+	+	+	+	+	—	±	±	±	±	+	+	+	±	±	+	+
60 per cent alcohol (5 minutes)	—	—	—	—	—	+	+	—	—	—	±	—	—	—	—	—	—	—	—	—	—	—	—	—

*This method was used with 1 minute staining, 1 minute in iodine, and 30 seconds in safranin as a counter-stain.
†Preparations lost.

TABLE 7.—REACTION TO THE GRAM STAIN OF VARIOUS CULTURES STAINED IN TRIPLICATE AND DECOLORIZED EITHER WITH 95 PER CENT ALCOHOL OR WITH ACETONE.*

METHOD AND DECOLORIZING AGENT	CULTURE NUMBER																							
	$M4_2$	D_1	G_2	D_6	X_4	D_8	$X1_1$	$W5_2$	$P31_1$	DS_1	116	213	40	148	154	37	90	91	195	149	201	382	155	97
Ammonium oxalate method (Hucker) with acetone	+	+	—	—	—	±	±	±	+	±	+	±	+	+	+	+	+	+	±	+	±	±	+	+
	+	+	—	—	—	±	±	+	+	+	±	±	±	+	+	+	+	—	+	+	±	+	+	+
	+	+	—	—	±	—	±	±	+	+	+	+	±	+	+	+	+	+	+	—	+	+	+	+
Ammonium oxalate method (Hucker) with alcohol	±	+	—	—	—	+	+	+	+	+	+	—	±	±	+	+	±	+	+	—	±	±	±	+
	+	+	—	—	—	+	+	+	+	+	+	—	±	±	+	+	+	+	+	—	+	+	±	+
	+	+	—	—	—	+	+	+	+	±	+	—	±	±	+	+	+	+	+	—	±	+	±	+
Anilin method (Buchanan) with acetone	+	+	—	—	—	±	—	—	+	±	+	—	—	+	+	+	+	+	+	+	+	+	+	+
	—	+	—	—	—	+	—	—	+	—	—	—	—	±	±	—	—	—	—	±	±	—	—	+
	—	+	—	—	—	+	—	—	+	—	—	—	—	+	—	—	—	—	+	+	+	+	+	+
Anilin method (Buchanan) with alcohol	±	+	—	—	—	+	—	±	±	+	+	+	±	+	+	+	+	+	+	+	+	—	+	±
	+	+	—	—	—	—	±	±	+	+	+	±	±	+	+	+	+	±	+	—	+	—	+	—
	+	+	—	—	—	—	±	±	+	±	+	±	±	±	+	+	+	±	+	±	+	—	+	—

*All preparations were in stain 1 minute, in iodine 1 minute, in decolorizing agent 1 minute, and 30 seconds in safranin as a counter-stain.

a decolorizing agent. Additional data are needed on this point, however, for the reagent certainly gives very acceptable results in the hands of different investigators and is being quite widely used at present. Burke (1921) and Kopeloff and Beerman (1922) particularly recommend its use. It is especially valuable now that it is difficult to obtain pure grain alcohol and to distribute the latter among students in a laboratory.

EFFECTS OF VARIOUS COUNTER-STAINS ON THE RESULTS OF THE GRAM STAIN

Various phases of the Gram technic have been compared above as to the constancy of the results which they give, but the matter of counter-staining has not been discussed. This matter in fact has not been given great attention in the literature, altho at the present time it is possible to find numerous bacteriologists who realize that the results may be decidedly influenced one way or the other by the choice of counter-stains.

To investigate this point, six different counter-stains were selected, and slides bearing preparations of the same 24 cultures mentioned above were stained with the same technic used in the preceding work and then counter-stained for 60 seconds with one or another of the six dyes. As in the work on decolorization, triplicate slides were stained in each case and the results listed separately in the table. The results are given in Table 8 for the ammonium oxalate method. The anilin oil method of Buchanan also was used in a parallel series of tests; but the results were so similar to those listed in Table 8 that they are not given.

By studying Table 8 it will be seen that there are two different sorts of discrepancies brought out by the results. In the first place, the individual slides sometimes fail to show clear-cut reactions and could not be recorded as either definitely Gram-positive or Gram-negative. Sometimes this was because stained and decolorized organisms were both present in about equal numbers, and sometimes it was because all of the organisms were partly but not wholly decolorized. These cases are recorded in the table by a $\pm$ sign. Another sort of discrepancy that can be observed in this table is the failure of the parallel slides to agree in their reactions. In recording discrepancies of this kind cases were disregarded where one of the three slides was marked $\pm$ and the other two either positive or negative. In other words discrepancies between the parallel slides were considered only

TABLE 8.—REACTION TO THE GRAM STAIN OF VARIOUS CULTURES STAINED IN TRIPLICATE AND COUNTER-STAINED WITH DIFFERENT DYES.*

COUNTER STAIN	CULTURE NUMBER																								
	$M4_2$	D_1	G_2	D_6	X_4	D_8	$X1_1$	$W5_2$	$P31_1$	DS_1	116	213	40	148	154	37	90	91	195	149	201	382	155	97
Safranin, 1:10 aq. sol.	−	+	−	−	−	+	+	+	±	−	+	−	−	±	±	±	±	−	−	−	±	±	±	−
	−	+	−	−	−	+	+	+	+	+	+	−	±	±	+	+	+	±	+	−	±	±	±	±
	−	+	−	−	−	+	+	+	+	±	+	−	±	±	±	+	+	±	+	−	±	±	±	±
Pyronin, 1:10 aq. sol.	+	+	±	−	−	+	+	+	+	±	+	±	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	−	−	−	+	+	+	+	±	+	±	+	+	+	+	+	±	+	+	−	+	+	+
	+	+	−	±	±	+	+	+	+	±	+	±	+	+	+	+	+	±	−	±	−	+	±	+
Bismarck brown, aq. sol.	±	+	−	−	−	+	±	±	+	±	+	−	±	+	+	+	+	+	±	±	±	±	+	+
	+	+	−	±	−	+	±	+	+	+	+	−	−	+	+	+	+	+	+	±	±	±	+	+
	+	+	−	−	−	+	±	±	+	±	+	−	−	+	+	+	+	±	+	±	+	±	+	+
Fuchsin, 1:10 aq. sol.	−	+	−	−	−	−	±	±	+	±	+	−	±	−	±	+	+	+	±	−	±	±	+	+
	−	+	−	−	−	+	±	+	+	+	+	+	−	−	−	±	±	±	−	−	±	±	±	+
	−	+	−	−	−	+	±	±	±	±	+	−	−	+	±	+	+	+	±	−	±	+	+	+
Eosin, 1:10 aq. sol.	±	±	−	−	−	+	+	+	+	±	+	−	+	±	±	+	±	±	−	−	−	±	−	+
	+	+	−	±	±	+	+	±	+	+	+	±	±	−	−	±	+	±	±	−	±	±	+	+
	−	+	−	−	±	+	+	+	+	+	+	+	±	±	+	+	+	±	±	±	±	±	−	±
Carbol fuchsin, 1 per cent in 5 per cent phenol	−	+	−	−	−	−	−	±	±	−	+	−	+	−	−	+	±	±	±	−	±	±	−	−
	±	+	−	−	−	±	±	±	−	−	+	+	+	+	+	+	±	−	+	−	±	−	±	+
	+	+	−	−	−	±	±	+	+	±	+	−	+	+	−	−	±	+	±	−	−	+	+	±

*The ammonium oxalate method (Hucker, 1922) was used throout, with 1 minute staining, 1 minute in iodine, 1 minute decolorization, and 30 seconds in counter-stain. Similar results were obtained in a parallel series with Bruchhaus's method.

when at least one of them was definitely positive and at least one definitely negative.

It will be observed that actual discrepancies of this last-mentioned sort are quite rare in the case of the first three counter-stains listed in the table, only two occurring in the case of safranin, two in the case of pyronin, and none in the case of Bismarck brown. In the case of fuchsin, however, three were observed; in the case of eosin, four; and in the case of carbol fuchsin, ten. Turning now to the consideration of those slides which failed to give clear-cut reactions as indicated in the table by ± signs, it will be seen that safranin gave 24 such indefinite results; pyronin, 13; Bismarck brown, 20; fuchsin, 23; eosin, 27; and carbol fuchsin, 20.

Another point to be noticed in these results relates to the number of organisms in each case which are regarded as negative. One of the chief objections to certain counter-stains is that they are so powerful in their action that they tend to decolorize some of the Gram-positive organisms. The best counter-stain then, should be the one giving the smallest number of Gram-negative results. By going over Table 8 and counting as Gram-negative in each case those organisms which were distinctly negative on one of the triplicate slides and not definitely positive in any of the three cases, it will be observed that 8 of the cultures were negative in the case of safranin, 4 in the case of pyronin, 5 in the case of Bismarck brown, 11 in the case of fuchsin, 7 in the case of eosin, and 8 in the case of carbol fuchsin.

Suming up these findings it would appear that pyronin and Bismarck brown are the best counter-stains, while eosin and safranin are fair substitutes. Another matter to take into account, however, is the color of the counter-stain, as it should be one that contrasts well with the color of the Gram-positive organisms. On this account Bismarck brown is not quite as satisfactory as the others, and eosin is often unsatisfactory because it does not stain sufficiently deep. It must be recognized, nevertheless, that these data are quite meagre and undoubtedly safranin and fuchsin will continue to be used widely by investigators who are accustomed to use them for this purpose. The authors must confess to a personal preferance for safranin.

COMPARISON OF THE CONSTANCY OF THE AMMONIUM OXALATE AND ANILIN OIL METHODS

Another point investigated in the present work was to compare the constancy of two of the best methods as shown by the preceding

work. The ammonium oxalate method (Hucker 1922) and the anilin oil method (Buchanan 1911) were again selected for the purpose of making this comparison. In each case safranin was used as a counter-stain, and the same length of time for the various procedures was adopted as followed in the work last mentioned.

For this work the four cultures which had proved the most variable to the Gram stain of the 24 used in the last work were selected for further study. These cultures were the ones designated $M4_2$, $W5_2$, 40, and 149. Fifty slides were prepared, each slide bearing in separate smears each of these four cultures. Twenty-five of these slides were stained by the ammonium oxalate method and 25 by the anilin oil method. In every case the slides were manipulated as nearly as possible the same way so far as concerns the time of the various procedures and other minor matters of technic. The results are given in Table 9.

It will be seen that altho there is a tendency for a little more constancy with the ammonium oxalate method than with the anilin oil method, nevertheless neither method can be considered anywhere near constant in its results. Inasmuch as variation in technic was prevented in this case as far as possible, it is evident that the variation in results in the case of these four organisms must be due to some characteristic of the organisms themselves rather than to a weakness in the method.

Further evidence of this same point was obtained by a second test. In this case one culture alone was selected, namely No. 40, which proved, if anything, the most variable of all four of those used in the work just mentioned. Two slides were prepared, each bearing 50 tiny drops made from an infusion of this culture. The drops were dried and stained as usual. One of these slides was stained by the ammonium oxalate method and one by the anilin oil method of Buchanan. Stained in this way it would be expected that all 50 drops in each case would be given exactly the same treatment; nevertheless, the results were as follows: In the case of the anilin oil method, 34 of the drops showed distinctly negative organisms, 13 doubtful, and 3 definitely positive; while with the ammonium oxalate method, 29 were negative, 19 doubtful, and 2 distinctly positive. It might at first thought be assumed that this variability was due to some of the drops being covered by some reagent a few seconds earlier than it reached other drops, but this is unlikely because of the

TABLE 9.—COMPARISON OF THE CONSTANCY OF THE AMMONIUM OXALATE (HUCKER) AND ANALIN OIL (BUCHANAN) METHODS.
Twenty-five identical preparations of each of four organisms stained by each method.

Anilin oil method	Culture No.	$M4_2$	—	—	—	—	—	±	—	—	—	—	—	—	—	±	—	—	—	—	—	—	—	—	—	—	—
		$W5_2$	—	—	±	±	±	±	±	±	±	±	—	±	±	±	±	—	±	±	—	—	—	±	+	±	±
		40	±	±	—	—	±	±	±	±	±	±	—	—	+	±	—	±	—	±	±	—	—	—	±	±	—
		149	±	+	+	+	+	+	+	±	±	+	±	—	+	+	+	—	+	+	+	—	—	+	+	+	+
Ammonium oxalate method	Culture No.	$M4_2$	—	—	—	—	—	—	—	±	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		$W5_2$	+	+	—	—	—	—	+	±	±	±	±	±	±	±	—	±	±	±	±	±	±	±	—	—	±
		40	±	—	—	±	±	—	—	—	—	—	±	+	±	—	—	—	—	—	—	—	±	—	—	—	±
		149	+	+	+	+	±	+	±	+	+	+	+	+	±	±	+	+	+	±	+	+	±	+	+	+	±

scattered location of those drops which stained differently from the majority.

Such results indicate an inherent variability toward the Gram stain in the case of certain organisms and suggest that it will probably be impossible by any technic that can be devised to obtain absolutely clear-cut distinctions between Gram-positive and Gram-negative organisms.

AGE OF CULTURE IN RELATION TO STAINING PROPERTIES

It is evident that the Gram stain is much more variable in its results than many observers realize. Workers who use the Gram stain as an aid in diagnosis, but make only one preparation and record the results as definitely positive or negative, may often be misled as to the true reaction of the culture under observation. Several factors seem to determine this variability only a few of which are possible to control, many of them in fact being still unknown.

Most prominent among the factors involved is the age of the culture. This possibility is quite commonly recognized among bacteriologists, as shown by the fact that in giving directions for making the Gram stain, authors often specify that the cultures must not be over some definite age, generally mentioning 24 hours. The common assumption is that Gram-positive organisms give the most vigorous reaction when they are very young, with a tendency to become negative as they grow older. This assumption seemed to be verified by looking over the records for a series of cocci studied by one of the authors at different ages of each culture examined. It was obserevd that many of the Gram-positive strains became doubtful or Gram-negative after three days of age.

The cultures on which these observations were made came mostly from pathogenic lesions and dairy products. When, however, a similar study was made of the records of a series of soil and manure cultures, mostly non-spore-forming rods, a very different tendency was observed. The results were so unexpected that they are tabulated in Table 10. The 21 cultures listed in this table were selected from a series of about 200 isolated from soil and manure. These were the only cultures in the whole series which showed a tendency to vary in their Gram reaction from day to day. In each case the preparations from each culture were made at the ages indicated from

a single agar slant and placed on a single slide. Thus the different preparations from each culture were all stained at the same time and by exactly the same technic. In the table a weak reaction is indi-

TABLE 10.—REACTION TO THE GRAM STAIN OF 21 SOIL ORGANISMS AT DIFFERENT AGES OF THE INDIVIDUAL CULTURES.*

CULTURE NUMBER	AGE OF CULTURE IN DAYS				SOURCE
	1	2	4	7	
1	—	+	+	+	Soil
2	—		—	+	Manure
3	+		—	—	Manure
4	—	—	±	+	Soil
5	±	—			Soil
6	—			+	Soil
7	—	±	+	+	Soil
8	—	—	—	+	Soil
9	wk.	wk.	+	+	Soil
10	—	±	+	+	Soil
11	—	±	+	+	Soil
12	wk.	—	+	—	Soil
13	—	+	+	+	Soil
14	+	—	+	+	Soil
15	—	wk.	wk.	+	Soil
16	—	—	—	+	Soil
17		+	—	—	Manure
18	wk.	+	+	+	Soil
19	—	+	+	+	Soil
20	—	—	+	+	Soil
21	—	+	—	+	Soil

*In each case the preparations from each culture were made at the different ages of a single agar slant and placed on a single slide. Thus the different preparations from each culture were all stained at the same time and by exactly the same technic. The ammonium oxalate method (Hucker) with safranin as a counter-stain was used.

cated by the abbreviation "wk." and a varying reaction by the sign ±. It is evident at a glance that the greater number of positive reactions were observed on the fourth and seventh days. Only two cultures in the list, Nos. 3 and 17, both from manure, were positive on the first two days and negative on the fourth and seventh days.

This daily variation in the Gram reaction not only tends to be in opposite directions in different organisms, but the same strain varies at different times in the strength of its reaction at any particular age. Thus no definite time can be determined at which any organism gains or looses its power to retain the violet stain. This effect of the age of the culture upon the Gram stain might be due to several causes, as for instance the action of autolysis or of the by-products of metabolism retained in the cell bodies. These by-products are dependent upon the available food supply and hence are undoubtedly variable from time to time even in the same culture.

Another factor to take into account is the possibility that these by-products may be ones that aid in the retention of the Gram stain. That substances of such a nature may be present is suggesetd by allowing preparations of Gram-positive organisms to be in contact with various solvents for different periods of time and then staining as usual. The writers thus found that hydrochloric acid, chloroform, ammonium hydroxide, and xylol all act on Gram-positive organisms in such a way that they lose their power to retain the violet stain.

It is brought out quite strikingly by the present investigation that in order to determine the Gram reaction of cultures a bacteriologist should stain each organism at least three times and make preparations of various stages of growth. In this way its general tendency in relation to the Gram stain can be observed. By referring to Table 9, for example, where 50 separate determinations of each of the four variable cultures are listed, it will be observed that cultures $W5_2$ and 149 are variable with a tendency to be positive, while cultures $M4_3$ and 40 are variable with a tendency to be negative. Such a statement as this means more than to try to call any one of these organisms definitely positive or definitely negative.

CONCLUSIONS

After a general survey of 19 different methods of Gram staining, it is very difficult to select any one method as superior to all the others. The four methods denoted in Table 1 as Jordan (1908), Buchanan

(1911), Atkins (1920), and Hucker (1921) seemed, in the present investigation, to give the most satisfactory results, and they are probably all equally efficient when fresh mixtures of the stain are used and the time of decolorization is kept under two minutes. In general laboratory use, however, where directions for the time of staining and decolorizing are often loosely interpreted and where it is not always practical to make up fresh solutions every time cultures are stained, all of these four methods are not equally satisfactory in every case. Two of them, namely the methods of Atkins and Hucker, use for mordants anilin sulfate and ammonium oxalate, respectively, neither of which has any harmful effect on the keeping qualities of the staining solution and are such efficient mordants that they allow ample latitude in the time of decolorization, hence, these two methods are regarded as especially useful.

A comparison of the various strengths of alcohol shows that little difference can be found between the results with 95 per cent or absolute alcohol, but that these two strengths give much more constant results than with alcohol containing more water. For this reason it is important, as pointed out by Burke (1922), that slides be carefully drained and blotted before putting on the alcohol so as to prevent diluting it.

Safranin, pyronin, Bismarck brown, and eosin were found more satisfactory as counter-stains than fuchsin at the strength used. Of them, pyronin and Bismarck brown gave the most constant results, but safranin was not much inferior in this respect and is often quite desirable on account of the sharp contrast it gives with the color of the Gram-positive organisms.

The authors feel that the Gram stain is a variable reaction even under the most carefully controlled conditions and no worker should base his results upon a single observation. It is recommended that, in order to determine the tendency of an organism with regard to the Gram stain, preparations of the culture be prepared at various stages of growth, from 12 hours to several days in age. All preparations should be made in triplicate on separate slides. In this manner a broader conception of the staining reactions of a culture may be secured than by the usual procedure. It is advisable, also, if possible, to stain the organism with more than one method in order to eliminate the possibility of a faulty technic.

Burke (1922) points out that the Committee on Bacteriological Technic should select carefully two cultures, one as a Gram-positive,

the other as a Gram-negative strain, taking care to choose for this purpose two organisms that lie close to the border line between these two groups. Burke claims that in this way better standardization of the Gram stain can be obtained than by trying to standardize the technic itself. This statement is undoubtedly true, and it is not impossible that two of the four cultures used in the work listed in Table 10 could be used for this purpose. Both the selection and distribution of such cultures will offer great difficulties; but it is, nevertheless, a matter to be given careful consideration.

Whatever is done in the way of standardizing the Gram stain, it must be definitely recognized that not all organisms are distinctly Gram-positive or Gram-negative; and that a large number should be placed in a class to be regarded as Gram-variable, altho a tendency one way or the other may be noted and recorded.

BIBLIOGRAPHY

Atkins, K. N.
1920 A modification of the Gram stain. *Jour. Bact.* **5**, *321–324*.

Buchanan, R. E.
1911 Veterinary Bacteriology. *Philadelphia*: *W. B. Saunders Co. 516 pp.* (See p. 103).

Burke, Victor
1921 The Gram stain in the diagnosis of chronic gonorrhea. *Jour. Amer. Med. Assoc.* **77**, *1020*.
1922 Notes on the Gram stain with description of a new method. *Jour. Bact.* **7**, *159–182*.

Claudius
1897 Méthode de coloration a la fois simple et contrastante des microbes. *Ann. Past. Inst.* **11**, *332–335*.

Committee on Bacteriological Technic
1922 An investigation of American gentian violets. *Jour. Bact.* **7**, *529–536*.

Eisenberg, Philipp
1910 Ueber neue Methoden der Tuberkel bacillenfärbung. *Berl. klin. Wochenschr.* **47**, *338–341*.

Eyre, J. W. H.
1915 Bacteriological technique. *Philadelphia*: *W. B. Saunders Co. 518 pp.* (See p. 108).

Friedberger, E. and Reiter, H.
1912 Die allgemeinen Methoden der Bakteriologie. *Kolle und Wassermann's Handbuch der pathogenen Mikroorganismen (Jena)* **1**, *293–554*. (See pages 360–363).

Gram, C.
1884 Ueber die isolirte Färbung der Schizomyceten in Schnitt und Trockenpräparaten. *Fortschritte der Med.* **2**, *185–189*.

Günther, Carl.
1887 Ueber die Mikroskopische Färbung der Wichtigsten pathogenen Bakterien mit anilin farbstoffen. *Deutsch Med. Wochenschr.* **13**, *471–475*.

Hucker, G. J.
1921 A modification and new application of the Gram stain. *Jour. Bact.* **6**, *395–397*. Also in *New York Agr. Exp. Sta. Tech. Bul. No. 84, 7–9*.
1922 Comparison of various methods of Gram staining (preliminary report). *Abs. Bact.* **6**, *2*.

Jensen, V.
1912 Hospitalstidende (See *Dairy Bacteriology*, by Orla-Jensen, p. 25.)

Jordan, E. O.
1908 General Bacteriology. *Philadelphia: W. B. Saunders Co. 557 pp.* (See p. 45).

Kisskalt, Carl
1901 Eine Modifikation der Gram'schen Färbung. *Centbl. f. Bakt.* 1 *Abt.* **30**, *281–387*.

Kopeloff, N. and Beerman, P.
1922 Modified Gram Stains. *Jour. Inf. Diseases*, **31**, *480–482*.

Kühne, H.
1888a Praktische Anleitung z. mikrosk. Nachweis der Bakterien im tierischen Gewebe. *Leipzig*.
1888b Ueber Färbung der Bacillen in Mallensknoten. *Fort. med.* **6**, *860–863*.

Kütscher
1894 Der Nachweis der Diphtheriebacillen in den Lungen mehrerer an Diphtherie verstorbener Kinde durch gefärbte Schnittpräparate. *Zeitschr. f. Hyg. u. Infektionskr.* **18**, *167–176*.

Leidy, Joseph
1919 Modification of Gram's stain for bacteria. *Abs. Bact.* **3**, *7*.

Löffler, F.
1906 Ueber die Veränderung der Pathogenität und Virulenz pathogenität und Virulenz pathogener Organismen durch Künstliche Fortsuchtung in bestimmten Tierspezies und uber die Verwendung solcher Organismen zu Schutzimpfungszwecken. *Deutsche med. Wochenschr. Jahrg.* **32**, *No. 31, 1240–43.*
1907 Neue Verfahren zur Schnellfärbung von Mikroorganismen, insbesondere der Blutparasiten, Spirochäten, Gonococcen und Diphtheriebacillen. *Deutsche med. Wochenschr. Jahrg.* **33**, *No. 5, 169–171.*
1910 Ein neues Anreicherungsverfahren zum farberischen Nachweise sparlicher Tuberkelbazillen. *Deutsche med. Wochenschr. Jahrg.* **36**, *No. 43, 1987–88.*

Lyon, M. W.
1920 Acetone as a decolorizer in Gram's staining method. *Jour. Am. Med. Assoc.* **75**, *1017.*

Mann, Gustav
1902 Physiological Histology. *Oxford: Clarendon Press, 488 pp.*

Mallory, F. B. and Wright, J. H.
1913 Pathological technique. *Philadelphia: W. B. Saunders Co. 507 pp.* (See p. 411).

Moore, V. A.
1912 Principles of Microbiology. *Ithaca: Carpenter & Co., 506 pp.* (See p. 129).

Novy, F. C.
1899 Laboratory work in Bacteriology. *Ann Arbor: Geo. Wahr. 563 pp.* (See p. 289).

Nicolle, Ch.
1895 Practique des colorations microbiennes (Méthode de Gram modifiée et méthode directe). *Ann. Past. Inst.* **9**, *664–670.*

Orla-Jensen, S.
1921 Dairy Bacteriology. *Philadelphia: P. Blakiston's Son & Co. 180 pp.* (See p. 25).

Scales, F. M.
1922 A new method for differential staining of bacteria. *Jour. Inf. Diseases.* **31**, *494–498.*

Stephan, S.
1909 Ueber eine besonders für Schnittfärbungen brauchbare Modifikation der Gramschen Färbungsmethode. *Centralbl. f. Bakt. 1 Abt.* **51**, *94–96.*

Stitt, E. R.
1911 Practical Bacteriology, Blood work and Animal Parasitology. *Philadelphia: P. Blakiston's Son & Co., 345 pp.* (See p. 31).

Stovall, W. D. and Nichols, M. S.
1916 Stabilized Gentian Violet. *Jour. Amer. Med. Assoc.* **66**, *1620–21.*

Tunnicliff, Ruth
1922 A simple method of staining Gram-negative organisms. *Jour. Amer. Med. Assoc.* **78**, *191.*

Unna, P. G.
1888 Die Entwicklung der Bakterienfärbung. *Centralbl. f. Bakt.* **3**, (See p. 193).

Weigert, C.
1887 Ueber eine neue Methode zur Färbung von Fibrin und von Microorganismen. *Fortschr. Med.* **5.**