TECHNICAL BULLETIN NO. 128 JULY, 1927

New York State Agricultural Experiment Station

Geneva, N. Y.

FURTHER STUDIES ON THE METHODS OF GRAM STAINING

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PUBLISHED BY THE STATION
UNDER AUTHORITY OF CORNELL UNIVERSITY

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TECHNICAL BULLETIN NO. 128

FURTHER STUDIES ON THE METHODS OF GRAM STAINING[1]

G. J. HUCKER AND H. J. CONN[2]

ABSTRACT

A detailed summary is given of Technical Bulletin No. 93 of this Station on "Methods of Gram Staining." The historical discussion of the Gram method of staining bacteria, given in the earlier bulletin, is reprinted and brought up to date.

Later work on the same subject verifies the conclusion of the earlier bulletin that "results with any particular organism may vary considerably, according to the technic used."

The earlier work indicated that some procedures are apparently more constant than others. One of the procedures found to be quite constant in its results (the ammonium oxalate method proposed by Hucker) has now been carefully compared with Burke's method and with Kopeloff and Beerman's modification of the same. No special advantage for any one of these three procedures has been found. It is plain, however, that with any one of them care must be used in the manipulation or Gram-positive organisms may be called negative, or vice versa.

Special emphasis should be given to the conclusion of the earlier bulletin 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."

It is pointed out that the conclusions drawn apply only to the use of the Gram stain in studying pure cultures of unknown organisms. The results do not apply to the technic when it is used for staining the organisms present in pus or other body discharges.

[1]This bulletin supersedes Technical Bulletin No. 93 of this Station, now out of print.

[2]The work as originally undertaken was planned primarily by the first named author. The investigations carried on since the earlier publication, however, were directed by the second author, who is also responsible for writing the present bulletin.

INTRODUCTION[3]

About forty years ago, Gram (1884)[4] 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" 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.

In 1923 the authors published a paper in which the various procedures for the Gram stain were discussed and a comparison of quite a number of them was made. The bulletin containing this paper is now out of print. It seems worth while to reprint certain sections of it and to add to them the information which has been gathered since the earlier publication, together with the conclusions that have been reached.

HISTORICAL

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	5 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

[3]The sections of this bulletin set in solid type are reprinted from Technical Bulletin No. 93; all other material is new.

[4]See Bibliography, page 31.

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 to 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

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.—VARIOUS MODIFICATIONS OF THE GRAM STAIN.—*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; add 3 to 8 drops 5 per cent solution of sodium carbonate and stain for 2 to 3 minutes.	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 detemined.

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

TABLE 1.—VARIOUS MODIFICATIONS OF THE GRAM STAIN.—*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 (1minute).**	———
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.**	———
Kopeloff and Beerman, 1922	1 gram methyl violet 6 B in 100 cc. water. Just before use mix 30 drops with 8 drops of 5% aqueous sodium bicarbonate (5 minutes or more).	Iodine 2 grams dissolved in 10 cc. normal NaOH, then made up to 100 cc. with water (2 minutes or more).	Acetone added drop by drop to tilted slide until no more color washes out.	0.1 per cent basic fuchsin (10 to 30 seconds).
Scales, 1922	1 gram Poirrier's blue dissolved by trituration in 1 cc. 95% alcohol added to 100 cc. of 5% phenol (20 to 30 seconds).	None.	2 grams safranin dissolved in 100 cc. 95% alcohol, added to 100 cc. acetone (3 to 4 minutes).	None (counter-staining accomplished during decolorization).
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.

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[5] has received considerable attention in the United States. The method requires a concentrated staining solution (5 per cent) and short staining periods (30 seconds).

This 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 (1920) 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.

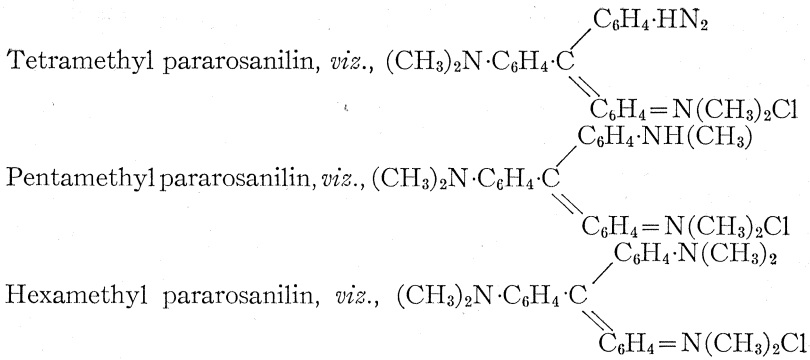
Various other minor modifications have appeared, as noted in Table 1, but the variations have usually been only in the concentration of the different solutions or special manipulation in the routine procedure of the staining.

Since the original version of Table 1 was drawn up, two new variations of the technic have appeared which have been added to the table as given in the present bulletin. These are the modifications of Scales (1922) and of Kopeloff and Beerman (1922). The technic of Scales differs quite radically from the other procedures in that its author employs an entirely different dye, a shade of anilin blue known as cotton blue, C4b (Poirrier's Blue), which he finds to work very similarly to gentian violet and without requiring the use of iodine after staining. The technic of Kopeloff and Beerman, on the other hand, combines parts of the procedures of Burke and of Atkins, respectively, in that the latter's iodine solution is employed in the technic of the former.

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:

[5]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 over Stirling's name.



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 designation 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 the 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.

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

REVIEW OF PREVIOUS STUDIES[6]

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

[6]Given in detail in Technical Bulletin No. 93.

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 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.

Methods Used in Testing the Various Procedures

In the work reported in Technical Bulletin No. 93, four different dye samples of the gentian violet group were employed, these samples varying considerably in their excellence. Three different organisms were used, *viz.*, *Bacillus cereus*, as a strongly Gram-positive organism; a fluorescent pseudomonad (probably *Ps. fluorescens*) as a Gram-negative; and a micrococcus isolated from cheese which had proved to be variable to the Gram stain, but with a tendency to be positive more often than negative.

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 greatly 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.

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 Preliminary Tests 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. 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.

From the standpoint of staining quality, the methods of Atkins and of Hucker proved superior to the other methods tested. The organisms were evenly stained, no precipitate was formed, and both the anilin sulfate and ammonium oxalate had 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 one year old with results apparently as satisfactory as with fresh solutions.

The further points investigated at the time of the earlier publication were effect of length of time of decolorization, effect of various strengths of alcohol in decolorization, comparisons of alcohol and

acetone as decolorizing agents, effect of various counterstains, comparison of the constancy of the ammonium oxalate and anilin oil methods, and relation of age of culture to its staining properties. The results of the investigation of these different points were as follows.

EFFECT OF LENGTH OF TIME OF DECOLORIZATION

In this work, the diphtheria organism was used as a test culture. It was found that by the two anilin oil methods selected this organism resisted decolorization for two minutes but not for ten, using 95 per cent alcohol as a decolorizing agent. By the anilin sulfate method of Atkins, it showed no decolorization in two minutes and only partial decolorization in 30 minutes. By the ammonium oxalate method it completely resisted decolorization for 30 minutes and partially so for three hours. It was evident from this that the period of decolorization permissable, without causing Gram-positive organisms to appear Gram-negative, varies with the mordant which is used.

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 IN DECOLORIZATION

The conclusions of Burke (1922) were verified in showing that when dilute alcohol is used for decolorizing there is less distinction between Gram-negative and Gram-positive organisms than in the case of strong alcohol (95 per cent or absolute). No apparent difference was noticed, however, between 95 per cent and absolute alcohol.

COMPARISONS OF ALCOHOL AND ACETONE AS DECOLORIZING AGENTS

Using an anilin oil method (Buchanan) and the ammonium oxalate method, a comparison was made between acetone and 95 per cent alcohol as decolorizing agents.

The results showed 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.

A further series of tests with 135 cultures of cocci led to the same conclusions in regard to the ammonium oxalate method and the following tentative conclusions were drawn:

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

A recent paper by Burke and Ashenfelter (1926) has taken exception to these conclusions and the authors have therefore investigated the matters further. The results are reported below (pages 24 to 28).

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

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.

The results of this work were given in Table 8 of Technical Bulletin No. 93 and the following observations and conclusions were drawn:

By studying the table 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 ± 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 ± and the other two either positive or negative. In other words, discrepancies between the parallel slides were considered only 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 counterstains 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 the table 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.

Summing 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 preference for safranin.

To this statement must be added the comment that others are equally justified in personal preferences of their own. It is plain that a bacteriologist may come to associate some color with the Gram-negative property to such an extent that it becomes difficult

to interpret results correctly if a counter-stain of a different color is employed.

COMPARISON OF CONSTANCY OF AMMONIUM OXALATE AND ANILIN OIL METHODS

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. 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.

There was found to be a tendency for a little greater constancy with ammonium oxalate than with anilin oil method, but the variation between different preparations of the same culture was quite extreme in either case. To find out whether this variation was due to differences in technic or to some characteristic of the organisms themselves, a second test was made.

In this case one culture alone was selected, namely, a coccus designated 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 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.

RELATION OF AGE OF CULTURE TO STAINING PROPERTIES

Realizing that the effect of age of the culture on the Gram reaction has been recognized for some time among bacteriologists, a series of observations were made and tabulated to illustrate the matter.

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 observed 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 of this study were quite unexpected. From a series of about 200 cultures isolated from soil and manure, it was found that 21 showed a tendency to vary in their Gram reaction from day to day. In each case, the preparations from each culture were made on the first, second, fourth, and seventh days from a single agar slant and placed on a single slide. Thus, the different preparations from each culture were stained at the same time and with exactly the same technic. When the results were tabulated it was evident at a glance that the greatest number of positive reactions were observed on the fourth and seventh days. Only 2 of the 21 cultures were distinctly positive on either the first or second days and negative on the fourth and seventh days. Both of these cultures were from manure and not from soil. This indicated that the tendency among soil organisms of Gram-variable reaction is to react more strongly as they grow older, whereas the organisms that have been more commonly studied by bacteriologists tend to show a weaker reaction in the older cultures.

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.

CONCLUSIONS OF TECHNICAL BULLETIN NO. 93

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 cultures used in this work could be employed 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.

SUPPLEMENTARY INVESTIGATIONS[7]

After publishing the previous bulletin on this subject, the success which other investigators have had with the method of Kopeloff and Beerman made it seem advisable to compare this method with the ammonium oxalate technic. Furthermore, it has been pointed out by Burke that the technic ascribed to him in Table 1 of Technical Bulletin No. 93 and used in the work reported in that Bulletin is not exactly as originally described by him. It seemed necessary, therefore, to repeat the comparison with his method, using the correct technic. This corrected technic is given in Table 1 of this bulletin.

The two procedures tested differ from each other and from the ammonium oxalate method in four respects, *viz.*, (1) a different dye solution was used in each procedure; (2) the method of Kopeloff and Beerman calls for an iodine solution different from the Lugol formula used in the other two procedures; (3) the ammonium oxalate method calls for alcohol as a decolorizer, the other two procedures acetone; and (4) Kopeloff and Beerman used basic fuchsin as a counter-stain, while the other two procedures call for a less powerful bacterial stain for this purpose, such as safranin or pyronin. These differences are shown graphically in Table 2.

It seemed advisable to compare these methods not only as described by the original author, but also as modified by using different combinations of the variations given in Table 2. It was felt especially important to investigate in this way the effect of acetone versus alcohol as a decolorizing agent. As stated above (page 16), alcohol had proved more satisfactory in the preliminary work. In this earlier work, however, the two decolorizing agents were compared only in the case of the ammonium oxalate and anilin oil methods. Since Burke's more recent findings show acetone to have decided advantages over alcohol, it was believed wise to repeat the comparison in both the Burke and the Kopeloff and Beerman methods.

The tests were made in the same way as before. Three of the organisms used in the earlier work, namely, *Bacillus cereus*, *Pseu-*

[7]Acknowledgment is made to Miss Mary A. Darrow and Miss Lida M. Thatcher who assisted in these investigations.

TABLE 2.—DIFFERENCES BETWEEN THE TECHNICS OF HUCKER, BURKE, AND KOPELOFF AND BEERMAN.

TECHNIC	CHEMICAL ADDED TO DYE SOLUTION	METHOD OF PREPARING DYE SOLUTION	IODINE SOLUTION	DECOLORIZER	COUNTERSTAIN
HUCKER	AMMONIUM OXALATE	1 PART SATURATED DYE SOLUTION MIXED WITH 4 PARTS 1% AMMONIUM OXALATE SOLUTION; MIXTURE KEEPS INDEFINITELY	LUGOL'S	ALCOHOL	SAFRANIN
BURKE	SODIUM BICARBONATE	1% DYE SOLUTION MIXED WITH 5% SODIUM BICARBONATE ON SLIDE	LUGOL'S	ACETONE	SAFRANIN
KOPELOFF AND BEERMAN	SODIUM BICARBONATE	1% DYE SOLUTION MIXED WITH 5% SODIUM BICARBONATE JUST BEFORE USING	2 GRAMS IODINE IN 10 CC. N/1 NaOH ADDED TO 90 CC. WATER	ACETONE	BASIC FUCHSIN

domonas fluorescens, and the Gram-variable coccus (No. 40), were used, placing a smear of each of the three organisms on the same slide. In interpreting results a stain was considered most satisfactory if it showed *Ps. fluorescens* as Gram-negative and the other two Gram-positive.

COMPARISON OF THE THREE RECENT PROCEDURES UNMODIFIED

The first step in this work seemed to be to make a direct comparison of the three methods, employing the technic described by the author in each case. The results are given in Table 3.

TABLE 3.—COMPARISON OF THREE PROCEDURES FOR THE GRAM STAIN, UNMODIFIED.

TEST NO.	REACTION OF THE THREE TEST CULTURES*								
	Burke technic			Kopeloff and Beerman technic			Hucker technic		
	B. cereus	*Ps. fluorescens*	Coccus	*B. cereus*	*Ps. fluorescens*	Coccus	*B. cereus*	*Ps. fluorescens*	Coccus
1	±	—	—	±	—	++	++	—	++
	++	?	+	∓	—	++	++	—	++
	±	—	—						
2	±	—	—	+	+	±	++	—	++
	±	—	—	++	+	+	++	—	++
	++	—	±	++	+	+	++	—	++
				++	—	++	++	—	++
				±	++	+	+	—	++
				—	++	+			
3							++	—	±
							++	—	±
							++	—	±
							++	—	±
							++	—	±
							++	—	±
							++	—	±
							++	—	±
							++	±	±

*In this table the symbols indicate as follows:
++A very strong positive reaction.
+ A fairly strong positive reaction.
? A doubtfully positive reaction.
± Positive and negative cells both present, positives predominating.
∓ Positive and negative cells both present, negatives predominating.
— A distinctly negative reaction.

Three tests were made in this series. In the first test, a few slides were stained by each technic; in the second test, several slides were stained by the Burke and the ammonium oxalate technics; and in the third test, a longer series of slides were stained by the Kopeloff and

Beerman technic in comparison with the ammonium oxalate method. These tests seemed to favor the latter technic for staining pure cultures of the organisms investigated. The Burke technic showed too much of a tendency for the positive organisms to stain negative, while the Kopeloff and Beerman technic stained *B. cereus* partly negative in the first test and *Ps. fluorescens* generally positive in the third test.

EFFECT OF VARYING THE COUNTER-STAIN IN THE KOPELOFF AND BEERMAN TECHNIC

The work just discussed suggested that basic fuchsin in the Kopeloff and Beerman technic might be too strong a counter-stain; and that when the technic was so manipulated (in test 3) as to give positive results with *B. cereus*, the negative organisms were not completely decolorized. Another test was planned, therefore, in which four different batches of basic fuchsin were compared with safranin and pyronin as counter-stains.

This test indicated rather better results with pyronin than with the other counter-stains, provided it is desired to learn whether a given organism is Gram-positive or Gram-negative, for with this counter-stain there is less tendency for the bacteria to appear partly positive and partly negative. Pyronin, however, is such a weak bacterial stain that the negative organisms do not stand out sharply; and if it is desired to have a technic that shows the negatives as clearly as the positives, pyronin is not a satisfactory counter-stain. Except for this slight difference between pyronin and the other counter-stains, the results were all practically alike. No difference was observed between the different batches of fuchsin, altho these samples represented various dyes of the basic fuchsin group. The results with safranin were the same as with fuchsin.

EFFECT OF VARYING THE DECOLORIZING AGENT

One of the principal differences between the ammonium oxalate technic and the other two methods under investigation is that the former uses alcohol for decolorizing, while the other two procedures call for acetone. To determine the effect of this difference, the procedures were repeated, both as originally called for and also with alternation of the decolorizing agent. In this work they were also compared with the same anilin oil formula as that employed in the earlier work. The results are given in Table 4.

This work made it very clear that acetone is a more effective decolorizer than alcohol. With acetone there was more of a tendency for the positive organisms to appear negative and with alcohol for the negative organisms to appear positive. It is interesting to note, however, that with the ammonium oxalate technic the best results were obtained with alcohol as a decolorizer, but that with the anilin formula and with the Kopeloff and Beerman technic the best results were obtained with acetone. In the case of the Burke procedure, the choice between the two decolorizers was a little more difficult to make. With alcohol, there was a slight tendency for *Ps. fluorescens* to stain positive; with acetone, for the coccus to stain negative. Of these two tendencies the latter is regarded as less misleading than the former, for *Ps. fluorescens* is such a distinctly Gram-negative organism that it should in no instance give a positive reaction, while the coccus used in this work is recognized as Gram-variable.

If the results are interpreted in this way, it is evident that each of the three technics under investigation gives the best results when used with the decolorizing agent specified by its author. In the earlier paper, where it was stated that the results favored the use of alcohol, conclusions were based almost wholly on the ammonium oxalate technic, the anilin oil method giving fully as acceptable results with acetone as with alcohol. In the present test, as stated above, it did better with acetone than with alcohol.

EFFECT OF VARYING THE METHOD OF APPLYING ACETONE

In both the technics of Burke and of Kopeloff and Beerman it is specified that the decolorizing agent be applied by flooding the slide instead of by immersion. This procedure, as stated by Kopeloff and Beerman, is as follows, "Add acetone (100 per cent) drop by drop until no color is seen in the drippings from the slide, which is slightly tilted. This usually requires less than 10 seconds and should be reduced to a minimum." It is sometimes a little difficult to tell just when to end this procedure, so an effort was made to determine the length of time that might be allowed for the decolorizing to proceed. In this work, while the slide was tilted, acetone was allowed to drop on it from a pipette, counting the number of drops as it fell. The smallest number of drops used was 30, the largest number 75. For comparison, the slides were also decolorized by immersion in acetone for two minutes, three minutes, and four

TABLE 4.—EFFECT OF VARYING THE DECOLORIZING AGENT IN THE THREE PROCEDURES GIVEN IN TABLE 3.

TEST No.	GRAM REACTION OF THE TEST ORGANISMS*											
	Burke technic			Kopeloff and Beerman technic			Hucker technic			Anilin formula		
	B. cereus	*Ps. fluorescens*	Coccus	*B. cereus*	*Ps. fluorescens*	Coccus	*B. cereus*	*Ps. fluorescens*	Coccus	*B. cereus*	*Ps. fluorescens*	Coccus
	Decolorized with Alcohol											
1	++ ++	— —	+ +	++ ++ ++ ++	— ? ? +	++ ++ ± +	++ ++ ++ ++ ++ ++	— — — — — —	∓ ∓ ∓ ? ++ ∓	++ ++ ++ ++ ++	∓ ± ± — —	++ ++ ++ ++ ±
2	++ ++ ++ ++	— — — —	++ ++ + +	++	++	++	+	+	+			
3	++ ++ ++ ++	— ∓ ± +	± ++ ++ ++	++ ++ ++ ++ ++	+ + + + ?	++ ++ ++ ++ ++	++ ++ ++ +	— — — —	+ + ± ±			

Decolorized with Acetone

4	++ ++ ++	— — —	± + ±	++ +	— —	± ∓	∓ ∓ ± ± ∓ ++ ++ ∓	— — — — — — — —	∓ ∓ — + — — ∓ —	+ + + +	— — — —	∓ + ± ±
5	++ ++ ++	— — —	— ± ±	++ ± ∓	— — —	± ∓ ∓	± ± ±	— — —	— ∓ ∓			
6	± ±	— —	— —	∓ ++ ±	— — —	± ++ ±	++ ±	— —	— ∓			

*In this table the symbols indicate as follows:

++ A very strong positive reaction.
+ A fairly strong positive reaction.
? A doubtfully positive reaction.
± Positive and negative cells both present, positives predominating.
∓ Positive and negative cells both present, negatives predominating.
— A distinctly negative reaction.

minutes, respectively. In this work both the Burke and the Kopeloff and Beerman procedures were followed, as well as a modification of the Kopeloff and Beerman technic in which the ammonium oxalate staining formula was used instead of the Burke solution with sodium bicarbonate. The results are given in Table 5.

In this test *Ps. fluorescens* stained positive by the Burke technic when less than 50 drops of decolorizer were used; by the Kopeloff and Beerman technic, when less than 60 were used; and following the ammonium oxalate formula, when less than 75 drops were employed. With the two former procedures, the coccus showed a tendency to be negative as soon as enough decolorizer was employed to bring out negative results with *Ps. fluorescens*. When the decolorizing was done by immersion, over three minutes seemed to be necessary to decolorize *Ps. fluorescens*.

This test was not repeated and standing alone is of little value. It merely suggests that the procedure of decolorizing is a very delicate one when used to distinguish between Gram-negative and weakly Gram-positive organisms. When following the procedure of Burke or that of Kopeloff and Beerman for pure cultures of bacteria, it is very difficult to be certain just when to stop the decolorization and one should make a series of determinations on an organism before deciding definitely whether to call it Gram-positive or Gram-negative.

CONCLUSIONS

The principal conclusion of Technical Bulletin No. 93, namely, that the Gram stain is a variable reaction, has been still further confirmed by later work. Further evidence along this same line has been furnished by Churchman (1927) who asserts that certain organisms, like *Micrococcus freudenreichii*, are stable Gram-positive organisms, while others, like *Bacillus anthracis*, are unstable. He concludes that with some procedures the latter organisms may be found uniformly positive, but that slight variations in the technic may cause them to appear negative. Such instances as this are of common observation to the writers, but in addition it has been found that some organisms vary in their Gram reaction even tho stained by identically the same technic.

In the case of these latter organisms the effect of slight variations in the technic is especially pronounced and it is practically necessary with them that some definitely specified period of staining and

TABLE 5.—EFFECT OF VARYING THE METHOD AND AMOUNT OF DECOLORIZATION IN TECHNICS OF BURKE AND OF KOPELOFF AND BEERMAN.

METHOD OF DECOLORIZATION		GRAM REACTION OF THREE TEST ORGANISMS*								
		Burke technic			Kopeloff and Beerman technic			Kopeloff and Beerman technic modified by use of ammonium oxalate		
		B. cereus	*Ps. fluorescens*	Coccus	*B. cereus*	*Ps. fluorescens*	Coccus	*B. cereus*	*Ps. fluorescens*	Coccus
By flooding, using the number of drops indicated	75 drops	++	—	±	++	—	∓	++	—	++
	60 drops	++	—	±	++	—	±	++	∓	++
	50 drops	++	—	±	+	?	±	++	∓	++
	40 drops	++	?	+	++	+	+	++	+	++
	30 drops	++	+	+	++	+	+			
By immersion for the number of minutes indicated	4 min.	++	—	±	++	—	++	+	∓	++
	3 min.	+	++	+	±	++	+	+	?	++
	2 min.	?	++	++	—	++	+			

*In this table the symbols indicate as follows:

++ A very strong positive reaction.
+ A fairly strong positive reaction.
? A doubtfully positive reaction.
± Positive and negative cells both present, positives predominating.
∓ Positive and negative cells both present, negatives predominating.
— A distinctly negative reaction.

decolorization be employed if results of any constancy are desired. It is particularly difficult to secure such constancy in the case of a technic where instead of specifying a definite length of time for decolorizing the directions are to decolorize until no more violet washes off the slide.

It is a question whether it is worth trying to obtain constancy in studying pure cultures of the Gram-variable organisms. In the writers' opinion, it is more important to learn whether an organism is almost invariably Gram-positive or whether it shows striking variations. In order to bring out this distinction, it is well to make several determinations upon the same culture, and to use at least two different staining procedures.

If results are interpreted in this way, it is futile to pronounce one technic better than any other for use in studying pure cultures. One test seems to show better results with one of the three procedures studied in this work and another test better results with one of the others. The writers are unable to say which is preferable for the purpose for which they have been studied and doubt if it is possible to make such a distinction.

It must be recognized, however, that in this work the Gram stain was used for making determinations on unknown pure cultures, and that the conclusions drawn do not apply to its use for other purposes. Burke, on the other hand, proposed his technic for diagnosing between positive and negative organisms in discharges from the body which may be so acid in nature as to interfere with the Gram stain unless some methods of correcting the reaction be employed, such as suggested by Burke and later employed by Kopeloff and Beerman. Thus employed, the Gram stain is wanted to make a sharp distinction between the strongly Gram-positive organisms and those that are strongly Gram-negative, and there is no need of recognizing the Gram-variable group. For such a purpose, constancy is highly desirable; and the writers are willing to grant that this may be secured, as claimed by Burke, much more effectively thru use of a staining solution that has been rendered alkaline.

It is recommended, therefore, that in studying unknown pure cultures, more than one of the recent modifications of the Gram stain be employed in order to compare the results obtained. When using the Gram stain in pathological work for diagnostic purposes, however, one should employ (with as little variation as possible) some technic that has been found to give the desired distinction.

The present investigation has not taken up the use of the Gram stain for the latter purpose as there seems no reason for questioning Burke's conclusions in the matter.

Finally, it must be urged that all authors publishing results depending in whole or in part on the Gram stain describe their staining method in considerable detail. In many a laboratory there may be some modification of the Gram technic which has been used without change for so long as to be more valuable to those who have employed it than any of the procedures preferred by the present writers. There is no need of such laboratories adopting a new technic, but their results will have more significance in other quarters if the exact steps of the staining procedure are published.

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