METHODS OF PURE CULTURE STUDY

PROGRESS REPORT FOR 1918 OF THE COMMITTEE ON THE DESCRIPTIVE CHART OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

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I. THE DESCRIPTIVE CHART AND ITS PURPOSE

Since 1905 there has been in existence, with occasional modification, a chart endorsed by the Society of American Bacteriologists for recording the characteristics of bacterial cultures. Recent literature, however, indicates that some confusion exists as to the purpose of this chart. This confusion was well illustrated at the 1917 meeting of the Society, the discussion called forth by the report of this committee showing that a number of bacteriologists have ideas as to the object of the chart different from those held by the present members of the committee. The confusion is natural, for each change in the membership of the committee has caused the chart to develop along slightly new lines; and even the early members of the committee have gradually changed their ideas as the result of later experience. It seems desirable, therefore, to restate the purpose of the descriptive chart.

The first bacteriologists to devise descriptive charts did so on individual initiative and apparently had in mind merely the concise description of their cultures preliminary to publication. An examination of the cards of Gage and of H. W. Conn[1] suggests that nothing further than this was expected of these two charts. Later, however, it was felt that uniformity in the cards used by

[1] For a description of these cards the reader is referred to a paper by H. A. Harding (1910).

different workers would make it possible to compare the results obtained in different laboratories. The Society, therefore, appointed a committee to prepare an official chart, and in its report this committee expressed itself as follows:

> The past literature of bacteriology abounds in such imperfect descriptions of organisms as to make their grouping, according to any system, impossible. This fact calls for the adoption of some scheme to which all descriptions shall conform, in order that no essential character shall be overlooked. The accompanying card is proposed for the recording of the characters of an organism. . . . . The cards can be filed like catalog cards, and arranged in accordance with the group number, thus bringing similar organisms together and rendering comparison easy.

This seems to imply that the chief objects intended to be served by the chart are first to secure uniformity in published descriptions and second to facilitate the grouping of bacteria according to their characteristics. Recent literature, however, reveals the existence of an idea not expressed in this report: namely, that the group number on this official card furnishes a more satisfactory method of characterizing an organism than its identification with a specific name occurring in the literature. The idea probably had its origin in the form of group number introduced with the Society card. Gage and Conn had both used a group number, but their group number consisted merely of four digits which could have suggested to no one that it constituted a complete description of a species. The Society group number, however, was simplified by leaving off those digits that indicated form and motility, replacing them with the genus symbol (according to Migula), which was placed before the series of digits. That this use of the genus symbol was not considered by the first committee to convert the group number into a method of characterising species is shown by the following statement which they made in regard to the group number:

> This system readily enables organisms having similar characters to be brought together and grouped about some central organism or type.

The only hint in this report that the group number might replace a specific name occurs a little later when the statement is made that according to this method of expression "Bacillus coli (Escherich) Migula becomes B.212.33310." This statement suggests to the reader, especially if that reader be a beginning student in bacteriology, that the string of digits 212.33310 is equivalent to the specific term *coli*. It naturally led to the impression on the part of the part of certain younger bacteriologists that the group number is nearly all-sufficient in describing a new species.

This point of view was strengthened by a paper from a member of this committee (Harding, 1910) on *Ps. campestris*, showing this organism apparently to have a constant group number. This work did not show that no other organism has the same group number, nor that all other organisms are so constant in physiological characteristics as is this particular species, nor indeed that this organism would have given such constant results if the technic had been varied; but there has been some tendency on the part of other bacteriologists to read into the paper a conclusion of this nature. The statement has even been made in one recent paper that the group number obviates the necessity of bacterial names.

On the other hand, various investigators, using other species of bacteria, have one by one criticised every point in the group number except that which refers to spore-production, showing that the same culture may give different results each time the tests are repeated. Futher criticisms have arisen from the biometric studies of bacteria, such variations having been observed among the different strains of a single species that no characterization of a species can be considered valid until the mean is determined around which the different strains vary.

These criticisms have sometimes been met by the statement that if the technic were sufficiently perfected and standardized, each strain would always prove to have the same group number, even though that group number might not express the characteristics of the species under all other possible conditions. An objection to this argument has been pointed out by Breed (1914)

in his work on the nitrate reduction of *B. coli*. He shows that the use of a standard medium, instead of distinguishing sharply between organisms with positive reactions and those with negative reactions, is likely to bring about variable results for those which grow poorly in the medium adopted. Recent work of the committee, moreover, shows very plainly that (in regard to nitrate reduction and acid production at least) no standard medium can be adopted which will give consistent results with all kinds of bacteria.

In brief, then, it may be said that the descriptive chart provides a uniform and concise method of recording nearly all of the ordinary and some of the unusual observations concerning the morphology and activities of an organism. Of the many characteristics listed on the chart, about a dozen were selected by former members of the committee as being the most important and were collected into a numerical expression called the group number. This group number is merely an abbreviated method of recording these characteristics, and must not be regarded as a sole means of characterizing species. Its value as a means of characterizing cultures, or strains, or species depends not only upon the wisdom with which these particular reactions have been selected but also upon the accuracy with which each individual characteristic can be determined. In both of these respects any group number proposed at present would necessarily be imperfect: first, because the diagnostic importance of the various characteristics differs among different groups of bacteria; and secondly because the methods for making the determinations have never been perfected and the results are correspondingly inaccurate. The problems of the committee, therefore, include the selection of the characteristics of greatest diagnostic value and the elaboration of methods which shall minimize mistakes. The committee is now studying the methods for working out the old group number; but, as a more permanent improvement, a new group number is being planned, less arbitrary and more logical than the one now on the card.

In addition to these weaknesses arising from our imperfect knowledge of bacterial activities and the methods of determin-

ing them, the card has not been revised frequently enough to contain the more recently devised tests for special groups of bacteria. Hence it is coming to be less and less used as a means of publishing descriptions of bacteria. In other words, it no longer helps to secure uniformity in bacterial characterization, the purpose for which it was originally intended.

Meanwhile instructors in bacteriology have realized the advantages of some such method of concise description in familiarizing students with the characteristics of bacteria. Some have used the official descriptive chart; but this chart was never intended for instruction purposes and is not designed to be put in the hands of beginning students. As a result other instructors have prepared descriptive charts of their own. Each of these charts has its own advantages and is undoubtedly better adapted to instruction than the old Society card; but for obvious reasons it would be better if a uniform style of chart were used in all institutions. The present committee, therefore, was instructed to prepare a chart especially adapted to use in bacteriological instruction: A new folder was prepared in response to these instructions and was presented at the 1917 meeting. It is now in print and is available to any teacher who wishes to try it out in his classes.

As the descriptive chart is now chiefly used for instructional purposes, it seems wisest to emphasize this use of it; but the committee does not wish to imply that an improved form of chart would be of no use in research work. The society card, indeed, has proved well adapted to certain types of research work in which a general survey is desired of the bacterial flora of some particular medium, preliminary to a later, more intensive, study of the individual species.[2] For some of this work the newer chart, although intended primarily for instruction, may prove more useful than the old form; but the old card is still available for use in this type of work whenever it is desired. Better still, for this purpose, would be a new card more nearly in accord with modern methods of characterizing bacteria, and

[2] Preliminary flora studies of this nature are well illustrated by the work of Conn, Esten and Stocking (1906) and by that of Harding and Prucha (1908).

specially adapted to research work. The committee hopes to prepare such a card in the future; but before that is done more attention must be given to the value of the various characteristics and to the methods of determining them.

The 1917 report of this committee (Conn. et al. 1918) contained a discussion of methods of pure culture study, designed primarily to accompany the chart for instruction. Further work has been done on some of these methods during the past year. A year ago it was expressed as the desire of the committee that these methods "be adopted as standard methods at the 1918 meeting, after such changes have been made in them as the year's use shows to be necessary." This is no longer urged. With the modifications given in the following pages, they are the best methods now known to the committee, and their use in instruction laboratories is strongly recommended; but as the results of criticisms of the 1917 report, together with the committee's further work during 1918, it seems wisest to regard them as provisional methods. No action is therefore asked on these methods and the committee desires to do further work on them.

The following pages contain a progress report on those methods that have been investigated during the past year, namely: Gram stain, acid production, and nitrate reduction. Free criticism of all matters discussed in this report, as well as the methods outlined in the preceding report, will always be welcome.

II. THE GRAM STAIN

At the last meeting of the Society one or two members criticised the classification of bacteria according to their ability to take the Gram stain, stating that many of the methods of making this test give irregular results and that the methods given in text books vary greatly from one another. The committee was asked to look into the matter. Gram originally gave no definite length of time for treatment in the different fluids; but as the results of the staining depend greatly upon the duration of each treatment, various investigators have felt it necessary to specify

definite periods of time. Unfortunately, however, scarcely any two investigators have agreed in the periods specified.

From two of the members who particularly objected to the lack of agreement between present methods a statement has been obtained as to the technic giving best results in their laboratories. The two methods thus obtained differ considerably: one method calls for a comparatively concentrated stain, a short period of staining and long decolorization, the other for a weaker stain, longer staining period and short decolorization. The former method is less time-consuming and the gentian violet solution it is claimed deteriorates more slowly; but upon comparison of the two methods no further differences have yet been observed. Both methods have been found to give clear-cut distinctions between the Gram-negative and Gram-positive organisms thus far studied. The committee realizes the advisability of adopting a standard technic, but feels that no method should be made standard without more investigation than has so far proved possible. Both methods are therefore given as capable of yielding good results; and the criticism of the Society upon them is invited.

Method 1

Gentian violet solution ..... 1 minute
(Stain prepared by grinding 5 grams in 10 cc. of 95 per cent alcohol in a mortar. Add 2 cc. anilin oil, distilled water 88 cc. Filter. Claimed to keep 3 to 4 months.)
Iodine solution (Lügol's) ..... 1 minute
(As usual: Iodine 1 gram, potassium iodide 2 grams, water 300 cc.)
Absolute alcohol ..... 2 minutes
Counter-stain ..... 30 seconds
(10 cc. of saturated alcoholic safranin in 90 cc. water.)

Method 2

Gentian violet solution ..... 3 minutes
(Anilin oil 3 cc., absolute alcohol 7 cc., water 90 cc. Shake and filter through moist filter. Add 2 grams of gentian violet and allow to stand 24 hours.)
Iodine solution (Lügol's, as above) ..... 2 minutes
Absolute alcohol ..... 30 seconds

Counter-stain:

Either Fuchsin (one part saturated alcoholic solution to nine parts of water) ..... 30 seconds

or Bismark brown (2 per cent, dissolved in hot water and filtered) ..... 2 minutes

Whichever method is used there are certain points in the technic that are important and must be insisted upon, especially if the test is being made by a beginner. The medium used must be recorded. Only young cultures should be used (eighteen to twenty-four hours old, except in case of unusually slowly growing organisms). Films should be on cover slips, made with distilled water, and in the portions examined organisms should be only one layer thick. Gentian violet and iodine solutions should each be removed by merely draining off the excess and blotting, before applying the next solution. Cover slips should be kept in constant agitation while in the alcohol and should then be transfered without washing to the counter-stain.

III. THE PRODUCTION OF ACID

Four places in the group number are devoted to the fermentation of sugars and glycerin. In the 1917 report the committee recommended studying this reaction in standard peptone broth. There are various possibilities of error inherent in this method, and it is not applicable to all bacteria. Accordingly a study has been made of the sources of error and the simplest methods of overcoming them.

Sources of error in determining acid production

One of the chief sources of error in determining acid-production comes from the weakness of the common methods of detecting and measuring acid. A change in the titre of any medium as the result of bacterial growth is not necessarily in the same direction as the actual change in H-ion concentration. This makes the use of the titration method unsatisfactory for detecting acid production. This source of error, however, may be overcome by the use of the proper indicators if the student has

a good comprehension of the principles of H-ion concentration. A simple but satisfactory method of using these indicators and interpreting the results was given in the 1917 report (pp. 122–124). The significance of these indicators and their use is discussed in more detail in the following pages.

Certain other sources of error are not so readily eliminated. Among them is the use of media in which the organisms in question make poor growth. If an organism does not grow well in a medium it is not correct to state that it is unable to produce acid from the sugar present in that medium. Certain organisms do not grow well in ordinary broth because they require more nitrogenous matter and a suitable medium for them should be found before adding the carbohydrate upon which their action is to be studied. Others require a different initial reaction from that of ordinary standard media. Others grow well only on solid media and give consistently negative results if tested in liquid media, merely because of their poor growth.

Another equally serious source of error is the fact that many bacteria may cause two simultaneous biochemical activities, one tending to raise the reaction, the other to lower it. The resultant change in reaction depends upon which of these two processes predominates, and the production of acid may often be entirely masked by the opposite tendency. This may often be the cause of variation in the results obtained upon repetition of the test with the same culture; and it may even cause an acid-producer to escape recognition entirely.

Error may also arise from impurities in the sugars used. Glucose is very often present as an impurity in other sugars, especially in those which are difficult to purify, but has also been observed in samples of sucrose and lactose. The presence of glucose can be detected by growing in it a known glucose fermenter that does not attack the sugar in question. This test should be made in all accurate work.

Media recommended

On account of these difficulties, it proves impossible to recommend any one standard medium. The formulae recommended

(J. Bact., **3**, 115, 116) are merely for routine use when the growth requirements of an organism are not known. If the organism grows poorly in these media, the results of the test are to be disregarded.

In connection with preliminary invigoration, the committee has already recommended (J. Bact. **3**, 118) dividing the organisms into four series according to their preference for 37° or 25°, and for liquid or for solid media. It is now further recommended that all organisms of series III and IV (i.e., growing well on the surface of agar but not in glucose broth) be tested for acid production by inoculating onto the surface of beef-extract agar (instead of broth) containing the carbonaceous substance to be tested. Bacteria not growing well in glucose broth or in beef extract agar must be tested in some medium in which they do make good growth. For them is recommended varying first the peptone content and then the acidity of the medium. The medium to which the sugar, glycerine or other substance is added should differ as little as possible from these standard media.

Methods of detecting acid production

For the reasons above discussed acid production should not be detected by means of titration. An indicator should be used whose range of color change covers the initial reaction of the medium used or else is slightly to the acid side of the initial reaction. (Thus brom cresol purple, with the range of $P_H = 5.2$ to $P_H = 6.8$ is the most satisfactory indicator to use with media of initial reaction of $P_H = 7.0$) Litmus can be used, but the results obtained with it are not sufficiently clear cut.

Indicator media are especially valuable now that we have indicators which are not attacked by bacteria, with turning points at the useful part of the P_H-scale. Litmus and methyl red are of but little use for this purpose because they are decolorized by bacteria; but the sulphone-phthalein indicators are not acted upon by bacteria and are just as satisfactory when mixed with the media before inoculation as afterward. The

addition of the minute quantities of these indicators necessary does not ordinarily influence the growth of the bacteria, and in most cases its influence can be disregarded.

The use of saccharine broth or agar containing brom cresol purple gives very clear indication as to acid production from the sugar present, unless the organism produces alkalinity from the peptone. This possibility should always be investigated by the use of some indicator with a more alkaline range, such as phenol red or cresol red. In case alkalinity is produced in the broth or agar without the sugar, it must be recognized that the production of acid may not raise the H-ion concentration of the medium above its original reaction. Theoretically the only way to be sure of acid production in such cases is to use some medium in which the organism grows well without producing alkalinity; but as such a medium is ordinarily difficult or impossible to obtain, this procedure is generally out of the question. The method recommended is to use a non-saccharine broth or agar as a check, both saccharine and non-saccharine media containing those indicators whose ranges cover the P_H changes likely to occur, and to pronounce the organism an acid-producer provided the H-ion concentration is greater in the presence of the sugar than it is in its absence. A very handy combination of indicators for this test is a mixture of brom cresol purple and cresol red. They may both be added to the same medium, giving a solution that changes very slowly from purple to yellow through a long range (from about $P_H = 8.0$ to about $P_H = 5.0$) extending to a considerable distance on both sides of neutrality. The exact amount of indicator used in the medium is not important as long as the color obtained is distinct. A convenient strength has been found to be 1 cc. of a 1.6 per cent alcoholic solution to the litre of medium. When brom cresol purple and cresol red are mixed, neither indicator need to be used in quite such great concentration.

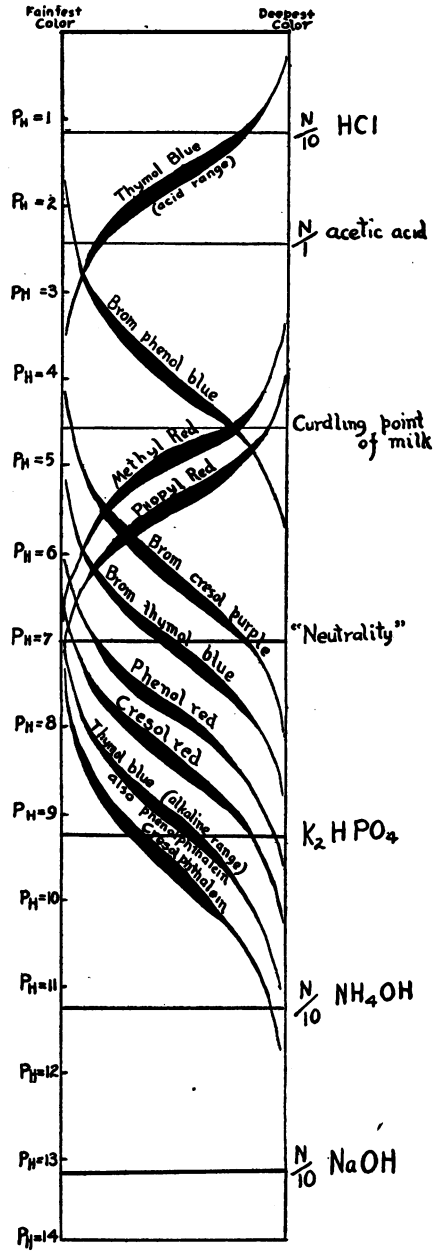

FIG. 1. COLOR CHANGES IN CLARK AND LUBS' INDICATORS. COMPARED WITH THE P_H VALUE OF CERTAIN IMPORTANT SOLUTIONS

Slightly modified from chart of Clark and Lubs

Relation of H-ion concentration to acidity

The above discussion will be readily intelligible to any reader who has an understanding of H-ion concentration and of the meaning of the symbol P_H. There would, indeed, be no point in discussing this subject further, if the research worker alone were concerned, because Clark and Lub's recent paper (1917) is readily available and treats the subject as fully and adequately as could be desired The new chart, however, is intended primarily for instruction and it is felt that teachers may desire a brief discussion of hydrogen-ion concentration for class-room or reference use. As there is at present no bacteriological textbook, so far as the committee know, that discusses the relation of acidity and reaction of media to H-ion concentration, and as copies of this report can be obtained from the secretary of the Society at about cost price, it seems well to summarize the subject here. Nothing new, however, is added in the following pages to what previous writers have said.

What is hydrogen-ion concentration?

The concentration of hydrogen-ions in a solution is used as a measure of acidity and alkalinity on the basis of the ionic theory. According to this theory, every salt, acid, or base, in aqueous solution, breaks up to some extent into "ions." Even pure water (H_2O) dissociates to a very small extent into H-ions and OH-ions. The H-ions bear a positive electric charge, the OH-ions a negative charge. Free H-ions are acid, free OH-ions are basic. In pure water both are present in equal amounts, thus neutralizing each other. Acids and bases dissociate to a much larger extent than pure water, the amount of dissociation depending ordinarily upon their strength. An acid dissociates into positive H-ions and negative ions consisting of the acid radicle. A base dissociates into positive metal ions and into negative hydroxyl (OH) ions.

The stronger acids (sulphuric and nitric, for instance) ionize to a greater extent than the weaker (acetic, for instance, and other organic acids). The more completely an acid ionizes in

solution, the greater the H-ion concentration of that solution. Therefore if different acids are dissolved in the same ratio of acid to water the H-ion concentration is an index of the strength of the acid. Similarly if different bases are dissolved in water in the same proportions, the OH-concentration is an index of the strength of the base.

If to a quantity of pure water (containing H and OH-ions) a drop of sulphuric acid be added, the mixture will contain H-ions, OH-ions and SO_4-ions. The number of free H-ions will be greatly increased because of the large number added in the drop of acid; but the number of hydroxyl ions will be decreased, because the change in ionic equilibrium will cause many of them to combine with free H-ions to form water. In other words, the H-ion concentration will be increased, the OH-ion concentration decreased. If, further, a larger quantity of the acid be added, the H-ion concentration will be still further increased, the OH-ion concentration still further decreased. It is evident, therefore, that H-ion increases and OH-ion concentration decreases as the acidity increases. Either may be used as an index of acid reaction; but H-ion concentration is preferred because it increases with increasing acidity.

When an acid is mixed with water containing other materials in solution, the matter is more complex. Various combinations are possible between the acid and the other materials in solution which prevent the H-ion concentration from being increased as much as it would have been if the same amount of acid had been added to pure water. Materials which thus repress the H-ion concentration of a solution are called "buffers." Organic matter in solution is especially likely to exert a buffer action. When buffers are present, the H-ion concentration of any solution will obviously be lower than it would have been with the same amount of acid added if no buffers were in solution. The actual reaction of such a solution is also lower, for only the ionized portion of an acid exhibits acid properties. The H-ion concentration, therefore, is a more correct index of the acid or alkaline reaction in a case like this than is the quantity of acid added.

When a base is added to water or to a neutral solution, the relation is exactly the reverse. The concentration of OH-ions is increased, that of H-ions is decreased. Either may be used as an index of alkalinity in terms of the ionic theory; but H-ion concentration is ordinarily used for the sake of uniformity. Hydrogen-ion concentration is inversely proportional to the dissociation of the base and therefore to the true alkaline reaction of the solution. Obviously a saturated solution of a strong base such as KOH has an exceedingly small H-ion concentration, but it is nevertheless measurable.

What is meant by P_H?

After deciding to use H-ion concentration as a measure of reaction the first necessity is to obtain some simple and concise method of expression. For this purpose the figures actually expressing H-ion concentration are quite unwieldy because of the extreme minuteness of H-ion concentration in weakly acid and in basic solutions. The H-ion concentration of pure water, for instance, has been shown to be 0.000,000,1 gram per litre, or in other words 0.000,000,1 normal (as a normal acid contains one gram of hydrogen per litre). A simpler method indicating this small quantity is by the logarithmic form of expression, i.e., 10^{-7} or log -7. This method of expression was adopted by Sörensen, who was the first to point out the great influence of H-ion concentration (as distinct from titrable acidity) upon biological activities. Sörensen used the symbol P_H^+ (or the simpler P_H) to represent this logarithm, without the minus sign; or using mathematical language, P_H is the logarithm of the reciprocal of the H-ion concentration expressed in grams per litre. Sörensen used this symbol to signify H-ion concentration. By this method of expression, the H-ion concentration of pure water is indicated by the formula $P_H = 7.0$.

Because P_H is an invert logarithm, it decreases with increasing H-ion concentration. Therefore, acid solutions have a P_H-value smaller than 7.0, basic solutions a P_H-value greater than 7.0. It has been found, for instance, that the P_H-value for $\frac{1}{10}$

normal HCl is 1.05, for normal acetic acid 2.4, while for $\frac{1}{10}$ molecular NH_4OH it is 11.2.

In interpreting P_H-values it must be remembered that adding 1 to the logarithm of a number is equivalent to multiplying the number itself by 10. For example, log 3 (i.e., 1000) = 10 × log 2 (i.e., 100). Similarly, to use an illustration that more nearly concerns us here, log −6 (i.e., 0.000,001) = 10 × log −7 (i.e., 0.000,000,1). In other words, a solution with a P_H-value of 6 has ten times the H-ion concentration of pure water ($P_H = 7$); and one with a P_H-value of 5 has ten times the H-ion concentration of a solution with a P_H-value of 6, or 100 times the H-ion concentration of pure water. Thus by a simple calculation it is possible to compute the relation between the H-ion concentrations of two solutions provided the difference in P_H-value is an integer. In case the difference in P_H-value is composed of an integer and a decimal, the relation may be learned by finding the number of which the decimal is the logarithm and giving it one more place to the left of the decimal point than there are units in the integer. An example will make this clearer:

For 1/10 normal HCl: $P_H = 1.05$
For normal acetic acid: $P_H = 2.4$

The difference is: 1.35
Now 0.35 = log 2.24,
But as the integer is 1, there must be two places to the left of the decimal point, i.e., 22.4 (approximately 22.5).
Hence tenth normal hydrochloric acid has approximately 22.5 times the H-ion concentration of normal acetic acid.

It is also possible to compute the OH-concentration for any solution of which the P_H-value is known, because there is a constant relation between the two expressed by the equation

$$P_H + P_{OH} = 14.$$

That is, P_{OH} may be determined for any solution by subtracting its P_H-value from 14. Thus for normal acetic acid ($P_H = 2.4$) the P_{OH} value is $14 - 2.4 = 11.6$.

Measurement of H-ion concentration

There are two different methods by which the H-ion concentration of a solution may be determined: the electrolytic method and the colorimetric method. The electrolytic method is generally the more accurate, and is applicable to a greater variety of solutions; but it is time-consuming and requires quite complicated apparatus. The colorimetric method is much simpler, is accurate enough for all bacteriological work and can be applied easily to any ordinary bacteriological culture medium.

The electrolytic method. The electrolytic measurement of H-ion concentration depends upon the fact that when a metal is in contact with an aqueous solution containing dissociated ions of that metal, there is an electromotive force set up between the metal and the solution, which varies according to the concentration of the ions of this metal in the solution. This electromotive force can be measured, and by means of the proper formula, the ionic concentration of this metal in the solution can be determined. Hydrogen acts like a metal in this respect when brought into electrolytic contact with an aqueous solution. This is accomplished by immersing a platinum electrode (platinum foil, coated with platinum black) in the solution and allowing a constant stream of hydrogen to bubble over the platinum. Because of the affinity of platinum for hydrogen, it takes up enough of the gas to act as a hydrogen electrode. The electromotive force established between this electrode and any aqueous solution with which it is in contact is inversely proportional to the H-ion exponent (i.e., P_H) of that solution.

The methods of measuring this electromotive force are quite complicated and are not adapted to the average bacteriological laboratory. All the average bacteriologist needs to know is that with the proper apparatus it can be measured and that the hydrogen ion exponent can be directly determined from it.

The colorimetric method. The colorimetric measurement of H-ion concentration depends upon color changes produced in certain substances (indicators) by varying acidity. Each indicator changes from one color to another between quite narrow limits of H-ion concentration, and between these two limits

every shade of the indicator corresponds to a definite P_H value. The best known indicators to the bacteriologist are litmus and phenolphthalein. Of these, litmus has little value in accurate H-ion measurements, partly because it is not a definite chemical compound, and partly because azolitmin, its chief component (even though chemically pure), varies so much in its color reactions according to the material present in the solution that little accuracy can be obtained with it. Phenolphthalein, on the other hand, gives very accurate results within the P_H-limits between which its color changes takes place; but this range (P_H = 8.0 to P_H = 9.6) is considerably to the alkaline side of neutrality. Bacteriological work, however, has special need of H-ion determinations near neutrality and in acid solutions.

In passing, it is of interest to notice that bacteriologists, in an empirical way, have realized for some time that the phenolphthalein color changes takes place in alkaline solutions. It has been found that bacteria grow better in solutions that are slightly acid to phenolphthalein, and it has lately become customary in this country to adjust media to 1 per cent normal acid to phenolphthalein. Bacteriological media have ordinarily contained Witte's peptone; and in 1 per cent solutions of this peptone, the above titre corresponds closely to the H-ion concentration of pure water (P_H = 7.0). This is not necessarily true of other peptones or of other media; but bacteriologists who have used peptone Witte have for some time adjusted their media practically to true neutrality.

The relation of different indicators to various points in the P_H-scale was studied by Sörensen as well as by others who have followed him. Most recently Clark and Lubs have furnished us (1917) with a beautiful series of accurate indicators whose sensitive ranges meet or even overlap and extend from P_H = 1 to P_H = 10. The relation of these indicators to H-ion concentration is shown in figure 1 and table 1. Of special interest to bacteriologists are the three indicators nearest to P_H = 7.0, i.e., brom cresol purple, brom thymol blue, and phenol red. Of these brom thymol blue is most useful in adjusting the reaction of media to neutrality, because it is yellow in acid solutions

and blue in basic solutions, passing through various stages of green between the points $P_H = 6.0$ and $P_H = 7.6$. At $P_H = 7.0$ it is grass-green, a shade that can be easily recognized by the eye after a little practice. As a result it is possible to obtain media that are essentially neutral, no matter what their composition may be, merely by adding sufficient acid or base so that brom thymol blue becomes grass-green when added to them.

It is plain that a rough idea can be obtained as to the H-ion concentration of any solution by simply finding which indicators give their acid color in it and which give their alkaline

TABLE 1

Color changes of Clark and Lub's indicators

INDICATOR	FULL ACID COLOR	FULL ALKALINE COLOR	SENSITIVE RANGE
Thymol blue (acid range)	Red	Yellow	1.2–2.8
Brom phenol blue	Yellow	Blue	3.0–4.6
Methyl red	Red	Yellow	4.4–6.0
Brom cresol purple	Yellow	Purple	5.2–6.8
Brom thymol blue	Yellow	Blue	6.0–7.6
Phenol red	Yellow	Red	6.8–8.4
Cresol red	Yellow	Red	7.2–8.8
Thymol blue (alkaline range)	Yellow	Blue	8.0–9.6
Phenol phthalein	Colorless	Red	8.0–9.6
Cresol phthalein	Colorless	Red	8.2–9.8

color. After a little experience with these indicators it is possible to do even better than this by inspection of the shade of color produced by whichever indicator or indicators are sensitive at the P_H-value of the solution in question. Accuracy, however, can be obtained only by actual comparison with the colors produced by the indicators in solutions of known H-ion concentration. For comparison Sörensen devised a series of standard solutions varying in H-ion concentration from $P_H = 1$ to $P_H = 13$. Clark and Lubs have proposed a different series of standards that are published in a more readily available place (loc. cit.). Of special interest is their standard corresponding to $P_H = 7.0$ (neutrality). This is prepared by mixing 5 cc. of $\frac{1}{5}$ molecular K_2HPO_4 with 3 cc. of $\frac{1}{5}$ molecular NaOH and dilut-

ing to 20 cc. with distilled water. Varying the proportion of these two solutions in the mixture increases or decreases the H-ion concentration as shown by table 1 of Clark and Lubs (1917, p. 26).

The difficulty of preparing such standard solutions of known H-ion concentration in the ordinary bacteriological laboratory has led Barnett and Chapman (1918) to devise a series of color standards that do not require accurate chemical adjustments. The standards that they propose are for phenol red, but they state that the method may be used for other indicators. Their method is to prepare two series of tubes, one series containing a

TABLE 2

Barnett and Chapman's color standards for H-ion determinations

Pair no. 1	5 cc. acid	+ 9 drops*	indicator; 5 cc. base	+ 1 drop indicator
Pair no. 2	5 cc. acid	+ 8 drops	indicator; 5 cc. base	+ 2 drops indicator
Pair no. 3	5 cc. acid	+ 7 drops	indicator; 5 cc. base	+ 3 drops indicator
Pair no. 4	5 cc. acid	+ 6 drops	indicator; 5 cc. base	+ 4 drops indicator
Pair no. 5	5 cc. acid	+ 5 drops	indicator; 5 cc. base	+ 5 drops indicator
Pair no. 6	5 cc. acid	+ 4 drops	indicator; 5 cc. base	+ 6 drops indicator

*In some laboratories it may prove simpler to use 10 cc. in each tube and to measure out the indicator solution in tenths of a cubic centimeter instead of in drops.

dilute acid solution, the other a dilute basic solution, adding decreasing quantities of the indicator to the acid series and increasing quantities to the alkaline series. The acid tube with the largest quantity of indicator and the alkaline tube with the smallest quantity of indicator form a pair (to be viewed together in transmitted light); the acid tube with the next largest quantity of indicator and the alkaline tube with the next smallest quantity form a second pair; and so on. This arrangement is shown in table 2.

It will be seen that the sum of the amount of indicator in the two tubes of each pair is always the same. Looking at the light through both tubes of any particular pair, the same color is obtained as when a solution of the proper H-ion concentration is viewed with an amount of indicator in it equal to the sum of

the quantity in the two tubes of the standard. The standard contains more indicator in the acid or in the alkaline tube respectively according to whether the pair of tubes is to represent the acid or the alkaline end of the range of the indicator.

Barnett and Chapman give the P_H-values corresponding to these successive pairs of tubes (using phenol red as indicator) as 6.9, 7.2, 7.5, 7.7, 7.9, and 8.1, respectively. Tested by a member of this committee they were found to correspond respectively to the P_H-values 7.1, 7.3, 7.5, 7.7, 7.8, and 7.9. Evidently the variation in the hands of different men is slight, probably less than in making up standard solutions of known H-ion concentration in the ordinary bacteriological laboratory. Such standards can be very quickly prepared even by a beginning student, and their use is therefore highly to be recommended.

The same method can be applied to brom thymol blue, thus covering a range more useful to some bacteriologists. The six pairs of brom thymol blue standards, prepared as given in table 2, correspond (as tested by a member of this committee) to the P_H-values 6.2, 6.4, 6.7, 6.9, 7.1, and 7.3, respectively. These two indicators together thus furnish standards covering the range from $P_H = 6.2$ to $P_H = 8.0$ with duplication at 7.1 and 7.3.

Why the titration method is illogical

The conventional method of adjusting the reaction of media or of determining the amount of acid produced by cultures depends upon titration to phenolphthalein. A computation is made of the per cent of normal alkali necessary to neutralize the solution, and the acidity is stated as equivalent to this per cent of normal acid. The assumption upon which this procedure is based is that the reaction of the solution is measured by the amount of base necessary to neutralize. This assumption is incorrect. Ten cc. of N/10 HCl and 10 cc. of N/10 acetic acid each require 10 cc. of N/10 NaOH to be neutralized, but the HCl is much more acid than the acetic acid.

In bacteriological media, moreover, there are nearly always present certain materials which have a buffer effect. Although

but weakly acid, they combine to a marked extent with the base used in titration and thus prevent the neutralization of the solution as rapidly as would be the case if no buffer were present. Peptone, for example, has a very strong buffer effect upon solutions in which it is present. As a result, in peptone solutions which are actually neutral, the titrable acidity may be quite high, because the NaOH used in titration combines with the peptone without greatly lowering the reaction of the solution. Hence titration is a very illogical method of determining the reaction of solutions containing peptone.

An example may make this clear. Witte's peptone is about neutral and its solutions are grass-green to brom thymol blue; but in a 1 per cent solution it titrates about 1 per cent normal acid to phenolphthalein. Difco peptone is more acid, giving a greenish yellow with brom thymol blue; but as it does not have such a marked buffer effect, it titrates lower than peptone Witte. As a result, now that Difco peptone is often used in America in the place of Witte peptone, it is not infrequent to find someone adding acid to it to bring it to 1 per cent normal to phenolphthalein, whereas alkali should be added to it to make it neutral to brom thymol blue. It should further be noticed that any peptone has practically the same H-ion concentration no matter what the concentration of the solution may be; but the titrable acidity is lower the more it is diluted. Hence the titration method leads to the error of adding acid to a 0.1 per cent solution of peptone to make its "reaction" the same as that of a 1 per cent solution.

A further fault of this titration method is that there is no sharp phenolphthalein neutral point. This indicator begins turning red at about $P_H = 8.0$ but does not reach its full alkaline color until about $P_H = 10.0$. Some bacteriologists titrate until its full color has appeared, others stop as soon as the first appearance of pink is evident, while still others try to use a point half way between these two limits. The result is considerable variation in the hands of different men.

All of these objections make the titration method entirely illogical for adjusting the reaction of media or for determining

the amount of acid produced by an organism. The colorimetric method of determining H-ion concentration is more logical, is of more significance, and is really more simple after the principles of the technic are once learned.

IV. THE REDUCTION OF NITRATES

The ordinary method of testing for nitrate reduction—as recommended in the last report of this committee (J. Bact., 3, 124)—is to grow the organism in a liquid nitrate medium and to test for nitrite after a definite length of incubation. The presence of gas is to be recorded if observed. On previous charts adopted by the Society the determination of ammonia was also called for, provision for making this determination having been made in the reports of the committees on water analysis of the American Public Health Association. It was distinctly stated in these reports, however, that ammonia may come from the peptone instead of from the nitrate, so that presence of ammonia in absence of nitrite does not necessarily indicate nitrate-reduction. This latter source of error, as has been pointed out by one member of this committee (Kligler, 1913), is greater than has been generally recognized by bacteriological writers. Hence no provision for the ammonia test is made on the latest chart recommended by the committee.

Omitting the ammonia test, however, opens up the possibility of error in another direction, for some organisms convert the nitrite into ammonia as rapidly as it is formed, so that its presence can scarcely be detected. This matter will shortly be further discussed by H. J. Conn and R. S. Breed in a paper (now in press) to appear shortly in this Journal. The nitrate-reduction test proves, indeed, to be a far more complicated matter than originally supposed. Absence of nitrite may mean any of the following things: (1) actual inability to reduce nitrate, (2) a medium so poorly adapted to the organism in question that growth is poor or lacking, (3) conversion of nitrite into ammonia as fast as produced, (4) assimilation of the nitrite-nitrogen (either as nitrite or as ammonia) as fast as

produced. Obviously a qualitative test for nitrite fails to distinguish between these four very different possibilities. A nitrite test accompanied by a quantitative ammonia test would undoubtedly prove an aid in detecting those organisms that reduce nitrate without an accumulation of nitrite; but as yet the committee has not worked out a quantitative test for ammonia simple enough for the bacteriological laboratory. Sometimes it is possible to obtain data of more significance by means of a qualitative test for ammonia, provided an ammonia-free medium is used containing no nitrogen other than the nitrate. In such a medium the only source of ammonia is the nitrate and its presence indicates nitrate-reduction even though nitrite be absent. As it is difficult, however, to obtain such a medium in which the majority of bacteria will grow, this method is realized to be of value only in special cases.

As a result the committee is unable at present to propose a standard method for the determination of nitrate-reduction. The provisional procedure recommended is as follows:

Inoculate first into nitrate broth and onto slants of nitrate agar. For this purpose use the standard formula for beef-extract broth (page 115 of 1917 report) with the addition of 0.1 per cent KNO_3, instead of the formula for nitrate broth given on p. 124 of the 1917 report. The richer medium is better adapted to pathogenic and to many other bacteria, and its greater viscosity aids in the detection of gas bubbles in case free nitrogen is produced. Test both the broth and the agar culture for nitrite by means of the reagents given on p. 124 of the 1917 report. (These reagents give the nitrite reaction as readily if poured on the surface of an agar slant as if added to a liquid culture). Presence of nitrite or of gas (indicated by foam in the broth or by cracks in the agar) shows the nitrate to have been reduced. A negative result does not prove that the organism is unable to reduce nitrates; in such a case further study is necessary, as follows:

In case the fault seems to lie in poor growth, search for a nitrate medium in which the organism in question does make good growth by means of the following modifications: increas-

ing or decreasing the amount of peptone; altering the reaction; adding some readily available carbohydrate. Presence of nitrite or gas in any nitrate medium whatever should be recorded as nitrate-reduction.

If the organism grows well and yet produces no nitrite or gas, the determination must be recorded as doubtful unless the organism can grow well in some synthetic medium containing no nitrogen except nitrate. It is recommended that such an organism be tested in a medium containing small quantities of phosphate, calcium, chlorine, etc., with KNO_3 as a source of nitrogen and sucrose as a source of energy and of carbon.[3] Such a medium generally allows good growth with an organism capable of utilizing nitrate and sucrose. Unfortunately neither glucose nor lactose can be used in this medium as a source of carbon and energy, for the ordinary "c.p." preparations of these sugars contain much ammonia. If the organism in question grows (even but slightly) on a synthetic medium of this sort, it should be tested for nitrite by the usual method and for ammonia by means of Nessler's reagent (comparing with an uninoculated tube as a check). The presence of nitrite, of ammonia (i.e., a more pronounced ammonia reaction than in check tube), or of gas indicates nitrate-reduction.

The production of gas (free N) from nitrate is not a very common one; but a considerable number of soil organisms have this power, and one should be on the lookout for it in studying soil bacteria. The agar slant test is ordinarily a sufficiently delicate test; but, if liquid media are used, more reliable results may be obtained by the use of fermentation tubes.

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[3] An illustration of such a medium which has proved satisfactory for some bacteria is: K_2HPO_4, 0.5 gram, $CaCl_2$, 0.5 gram, KNO_3, 1 gram, sucrose 10 grams, agar 12 grams, water 1000 cc.

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