Effect the alteration of pH has upon a basic and acid dye for use in a modified bacterial capsule staining technic

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5η Βαθμίδα

Νοέμβριος 1989
I wish to express my appreciation to Dr. B. B. Jeffers and Dr. D. H. Plotter for the advice given me during the course of experimentation. To Dr. B. B. Jeffers, whose invaluable suggestions on procedures and staining techniques made possible this study, I wish to express my utmost gratitude.
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Most observers contend that the majority of bacteria possess a capsule. However, this extracellular envelope is ill-defined and difficult to demonstrate in many species. The bacterial capsule has been correlated with the virulence of many organisms. This correlation brought the advent of much research, around 1900, pertaining particularly to the physico-chemical makeup of the capsule.

There were many staining methods devised at this time to demonstrate the capsule in different species. The organism of virulence concern in these staining procedures was diplococcus pneumoniae.

Holl was recorded his experience with old and new bacterial stains up to 1925 (21). He found that the methods of Ehrlich, test, Ruin, Macleod, Simon, and Kello and coneuron for staining capsules with aqueous solutions of eosin violet or crystal violet were unsatisfactory in that the preparations often dried and the method of Ehrlich, which involved the use of aqueous solutions of benzine, was impractical.

This published a method of capsular staining devised in 1905 (2). This method involved the
and is now used extensively as a routine method. The
Wiss procedure is comparatively simple but a certain
amount of experience and judgment is needed if a satis-
factory stain is to be secured.

Another staining method was used by Burgar to note
the morphology of the capsule with reference to Diplo-
occus pneumoniae and Streptococcus (1). His technic
required the use of various chemicals and moments. Al-
though the method was complicated, it was reliable.

Many methods for staining the bacterial capsule
were found to be satisfactory by various observers.
However, the majority of these stains were very intricate.
The purpose of this study was to note if a basic or acid
dye altered to a certain pH could be used in a practical,
simplified, and reliable capsule staining procedure.

A definite step in the introduction of a simple
capsule staining method was made by Dunton (12). The
staining material comprised many chemicals, but the
procedure took only 1 minute to complete. The fixing
and staining material used in this method was made up of
2% carbolic acid 1 cc., concentrated lactic acid 0.25-0.5 cc.
1% acetic acid 1 cc. and saturated alcohol solution of
basic fuchsin 1 cc., carbol fuchsin (old) 1 cc. The
acidic solutions used in this method suggest that the
mechanism involves simply the lowering of the pH of the
solution in question.
A capsule staining method involving basic fuchsin on the stain, and methylene blue in the counterstain was reported by Leifson (13). The capsule was stained i
and the organism was stained blue. Glass method (9) using India ink, demonstrated the capsule in solid. Still another technique, devised by Eggeling (2) utilizing Whitt's stain was used with fair results. Anthony (1)
reported one of the latest capsule stains in 1951. His procedure was very simple. To used 1% aqueous solution
of crystal violet as the stain and washed with 6% copper sulfate.

General nature of the capsule

The capsule is a viscous material which forms concentrated around many bacterial species. The ability to produce the capsular material is a hereditary characteristic which is lost when the culture undergoes phase
variation or dissociation.

Little is known concerning the influence of environment on capsule production. There are, however, some environmental factors which influence the close formation of the capsule. It has been shown that cultures of the shown type in the presence of certain substances (17-21°C) in presence of fermentable carbohydrolyc capsules are produced (15). The study (11)
found that the largest capsules produced by Lactobacillus

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enriching were obtained by cultivation for about 5 hours at 37° C. in a medium containing 1% peptone and 2% glucose. It was demonstrated also that encapsulation, for this species occurs under conditions unfavorable for growth and not when active proliferation occurs. Both unencapsulated organisms produce large capsules as young cultures. Young organisms of *Listerococcus innocuum* in solutions from tested large capsules, but these capsules degenerate in old ones (17). Vernon (16) also showed the capsule to be visible only in young cultures of various strains of *Listerococcus*.

The chemical nature of the capsule was studied with different species of bacteria. The capsule of a number of bacterial species consists essentially of a polysaccharide of high molecular weight. This polysaccharide may vary in chemical structure for different strains within a (species). The chemical difference of the capsular carbohydrate has been shown important values for immunological specificity of the different pathological types (3). Dormand (2) determined the structure of *Cocillus anthropus* to be different from a polysaccharide. It was made up of a tripeptide of d(-) glucaric acid.

**Chemical nature of dyes used**

A dye is defined as an organic compound which contains chromophoric and auxochrome groups attached to
benzene rings. The color is attributable to the chromophores and the staining property to the salt-forming auxochromes (6a).

Dyes are spoken of as basic and acidic but these terms do not refer to the actual hydrogen-ion concentration of the dye. Generally, the basic dyes are sold as salts of a colorless acid, and the acid dyes are sold as their sodium, potassium, calcium, or ammonium salts (6b).

Dyes are classified according to their similarity in chemical structure. Crystal violet (6b) is classed as one of the phenyl-ethyl dyes. This is a basic dye which has the chemical name of hexa-ethyl 2-methoxycin. Acid Fuchsin (6c) is classed as a phenyl nothume dye also. This dye has its acid character to the fact that it is a sulfonated (-SO₃H) derivative of basic Fuchsin.

Staining Mechanism

Many theories concerning the staining mechanism involved for bacterial systems has been propounded. Two theories which attempt an explanation of staining phenomena are presented here. The first theory is concerned with physical factors. It has been called the "adsorption theory" (10). According to this theory staining results when the dye is deposited and retains on surfaces as a result of physical forces.

The second theory is based on chemical factors (10).
The proponents of this theory state that the cation of the dye supposedly forms a salt with some reactive group of the stained material. These theories have many followers, but both are somewhat inadequate for the thorough explanation of the staining phenomenon.

Generalizations concerning the staining mechanism in techniques which involve several steps and the use of reagents are very difficult to derive and support. Other factors must be considered, for the chemical and physical theories are inadequate as they stand now. One of the big factors, recognized by Folin-Coeurier (20), which affects the outcome of a staining process is the pH. Using a mixture of acid plus basic dyes at various levels of the staining solution an approximate method for determining the isoelectric point of different staining solutions was shown also that staining with acid dyes is more effective at low pH levels, whereas the basic dyes are most effective within the alkaline range. Rella (14) also demonstrated quantitatively that the absorption of crystal violet, a basic dye, was greatest on the alkaline side of a section, and the reverse was true for acid staining. Other factors as going to the staining process have been observed, but they are irrelevant to this study.
MATERIALS AND METHODS

Strains of Streptococcus inolysicus, Streptococcus pyogenes, 
Haemophilus parainfluenzae, Haemophilus influenzae, and 
Haemophilus diphtheriae were chosen for study. These 
were selected from the culture stock in the 
Bacteriology Department, Montana State University. 
These strains had been kept on artificial media for approxi- 
ately one year. Three strains of the "near clean type 
Type VIII," and one Type IV were obtained from J. A. 
Hernandez. Before the start of experimentation the cys-
tological and cultural characteristics of these strains, 
with the exception of the pneumococcus, were checked and 
found to be accurate.

The medium most satisfactory for cultivating the 
organisms was 10 ml. of sterile beef serum broth on a 10 
ml. nutrient agar plate. The pH of the nutrient agar 
was determined to be 7.1 to 7.2 and the pH of the beef 
sirum varied from 6.3 to 6.5.

The organisms were transferred four successive times 
at 12 hour intervals before use. The preliminary pro-
cedure was employed because the carrying became 
evident. Young encapsulated organisms were used. In 
the first experiments the organisms were incubated at 
77° C., but since the results proved inconsistent at 
times, a suspension of 30° C. was tried and found to
be more satisfactory. The relative 'safety' coefficient is obtained by placing a small pan of water at the bottom of the incubator. This latter procedure proved to be of value in extending experimental time.

For each staining solution, a saturated alcoholic solution of crystal violet, and a saturated alcoholic solution of acid fuchsin. The crystal violet solution was prepared by dissolving 15 cc. of dye (10% dye content) in 100 cc. ethanol. The acid fuchsin was prepared by dissolving 7 cc. of dye (70% dye content) in 100 cc. of ethanol alcohol.

The pH of the saturated alcoholic solutions of crystal violet and acid fuchsin was determined electrometrically with a Bausch and Lomb, pH meter, the 2nd. solutions of each dye were individually altered in pH, using 0.01 N HCL or 0.01 N NaOH for lowering and raising the pH levels.

Samples of the crystal violet solution were settled by varying pH ranges of 10.0 to 1.0 using 0.01 N HCL to raise the pH and 0.01 N HCL to lower it. The acid fuchsin was altered through a pH range of 7.0 to 2.0 using the same and acid as above. 0.01 N HCL was used to lower the pH of both dyes from 2.0 to 1.0. The altered samples of each of the respective dye solutions was placed in a dark proof mess tube and the tubes were sealed. It is noted that it will be unfeasible to use a filter with
The continuation of the text...
13 to 2½ hours. As indicated, the staining solutions used in this part were the varying pH samples of the saturated alcoholic crystal violet. A 3 mm. loopful of serum from a tube containing the organisms was placed on a clean slide. The loop was flamed. A loopful of staining solution was spooned out and placed on the drop of serum. The mixture was allowed to stand on the slide for approximately 30 seconds and then mixed in a horizontal rotary motion with the loop so that an equal distribution of stain, about the size of a quarter, was obtained. It was noted that cross-hatching, and zig-zag actions on the stain made for a poor preparation. Stains were allowed to dry and observed around the periphery of the outer for the encapsulated organisms. This procedure was repeated for all organisms with the various solutions of stain. The experiment was repeated twice.

Part II.

The staining solutions used in this part were the varying pH samples of the saturated alcoholic crystal violet. The procedure involved in this part was modified to stain the organism dry, and wash the stain off with 20% copper sulfate. A 3 mm. loopful of serum from a tube containing the respective species of organism was placed on a clean slide. The drop of serum was spread on the slide in a rotary motion, with the loop, to about the size of a quarter. The smear was allowed to dry without
heat fixation. The slide was flooded with some of the sample of the solution of the crystal violet and allowed to stand 1 minute. The stain was washed off with 3% copper sulfate. It was then air-dried and observed under oil immersion. As in part I this procedure was repeated for all organisms used with the various solutions of the saturated crystal violet. The experiment was repeated twice.

Part III.

The organisms used were incubated at 31° C. for 10 to 24 hours. In this part the procedure was identical with that used in part I except that the various solutions of the alcoholic acid fuchsin were used.

Part IV.

The organisms employed in this part were also incubated at 31° C. for 10 to 24 hours. The procedure used in part II was employed. The various solutions of the acid fuchsin were used to stain the smear.
Table I indicates the results of the experimental procedure in Part I.

In this series of experiments the capsule was not
nepitively stained by the Niss method, which was
as a control. The capsule of all organisms used in the
procedure of Part I appeared in relief with the use of
the saturated alcoholic crystal violet at various dilu-
sions. However, the capsules possessed by Neobalilla
procumbens, and Neobalilla rimosae were more dis-
inctly stained by the method described than they were by
the Niss technique. Under the conditions of Part I this
washing procedure, on the whole, was not as effective as
the Niss method as regards the other micro- and sub-
species used.

Table II, from the results of the wax used in
Part II. The capsule, in all cases, with the wax
used, took on a positive stain. The Neobalilla ill took
a deep purple stain and the capsule at one side of
this blue halo surrounded by a distinct colored zone. As
compared with the Niss method it is possible we have to
be superior.

The results of the procedure used in Part III are
shown on Table III. It was observed that the Niss color-
sin solutions into poor cellular staining preparations at almost any pH level. Even the bacterial cell of all species appeared lacy between a pH range of 2.5 to 7.5.

In Table IV are recorded the overall results of Part IV.
Table I. Showing results of Part I.

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<tr>
<th>ORGANISM</th>
<th>pH 10.0</th>
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Legend:  
- = capsule not stained  
++ = capsule indistinctly stained  
+++ = fair capsule stain  
++++ = good capsule stain  
++++++ = very distinct capsule stain  
ii = negative capsule stain  
P = positive capsule stain
Table I. Concluded.

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Legend:  
- = capsule not stained  
++ = capsule faintly stained  
+++ = fair capsule stain  
### = good capsule stain  
#### = very distinct capsule stain  
$J$ = negative capsule stain  
$P$ = positive capsule stain
Table II. Concluded

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Legend:  
- = capsule not stained  
++ = capsule faintly stained  
# = faint capsule stain  
## = good capsule stain  
### = very distinct capsule stain  
- = negative capsule stain  
+ = positive capsule stain
Table III. Concluded

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<th>ORGANISM</th>
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Table IV. Showing the results for Part IV.

| ORGANISM         | pH 7.8 | pH 7.6 | pH 7.5 | pH 7.4 | pH 7.3 | pH 7.2 | pH 7.1 | pH 7.0 | pH 6.9 | pH 6.8 | pH 6.7 | pH 6.6 | pH 6.5 | pH 6.4 | pH 6.3 | pH 6.2 | pH 6.1 | pH 6.0 | pH 5.9 | pH 5.8 | pH 5.7 | pH 5.6 | pH 5.5 | pH 5.4 | pH 5.3 | pH 5.2 | pH 5.1 | pH 5.0 | pH 4.9 | pH 4.8 | pH 4.7 | pH 4.6 | pH 4.5 | pH 4.4 | pH 4.3 |
|------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| *Strep. homolycus* | +      |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |
| *Strep. influenza* | +      |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |
| *Pneu. IV*        | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      |
| *Pneu. VIII*      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      |
| *Pneu. VIII*      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      |
| *Pneu. VIII*      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      |
| *Klebsiella rhinos* | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      |
| *Klebsiella rhino* | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      |
| *Enterobichia col* | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      | #      |

Legend:  
- = capsule not stained  
+ = capsule indistinctly stained  
## = fair capsule stain  
### = good capsule stain  
#### = very distinct capsule stain

P = negative capsule stain  
P = positive capsule stain
<table>
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Plate 1.

Klebsiella rhinoscleroma (x3000), as demonstrated at pH 2.0 in procedure of Part II.
Plate 1
DISCUSSION

CH. IV

A. E. Stearn (10) noted that basic dyes cause an increase in hydrogen ion concentration when these dyes are added to a solution of typical protein. The acid dyes when treated with protein show the effect of decreasing the hydrogen ion concentration. Taking this point into consideration the pH of the dye solutions may have been altered consequent by the protein in beef serum. Relatively, however the pH of the staining solutions could be constant assuming the effect of the protein in beef serum to be stable.

The capsule stain in part I had the effect of a relief stain. *Streptococcus pneumoniae* and *Streptococcus pyogenes* at the pH levels of 2.0 and 2.1 displayed well defined capsules. As compared with the Kibbs method the capsule observed in these two organisms was easier to demonstrate. The capsule in the other species was stained better by the Kibbs method.

The procedure carried out in part II was a not satisfactory one. The one particular procedure, within a limited pH range, displayed a positive staining effect superior to the control method.

It is indicative that the pH values, at which the capsule stained (for most of the organisms used), falls
within a low range. No explanation as to why Streptococcus hemorrhicus and Streptococcus hemolyticus demonstrated a capsule stain within a varied pH range is offered.

Results using the saturated alcoholic acid fuchsin at various pH levels were definitely negative.

One of the initial purposes of these experiments was to note if a dye altered to a certain pH could be used in a practical, simplified and reliable staining procedure. The results are not decisive since one pH solution of the crystal violet or acid fuchsin dye could not be used satisfactorily to stain the capsule of all organisms.

Altering the pH of a saturated basic dye (crystal violet) does have an effect on the capacity of that dye to stain a capsule in the presence of certain pH at which the dye stains the capsule not effectively varies with the different organisms. The acid dye (acid fuchsin) was ineffective as a capsular staining material.
1. The effect of altering pH on a saturated basic and acid dye for use in a modified bacterial capsule staining technic was studied.

2. Two procedures for staining the capsule were advanced. In one method, the encapsulated organisms were stained as a wet serum preparation. In the second, the stain was flooded on a dry smear, and washed with 20% serum saline.

3. Apparently the most satisfactory procedure involved staining a dry smear with the particular pH solution of crystal violet and washing with 20% serum saline.

4. The results using the acid dye (acid fuchsin) were negative.

5. At certain pH levels of the crystal violet dye, the staining method used was superior to the acid fuchsin.

6. A certain impracticability of the capsule staining procedures was noted. One pH solution of either the crystal violet or acid fuchsin could not be used satisfactorily to stain the capsule of all organisms.

7. It is stressed that more encapsulated or units, exploring narrow pH ranges could be studied to implement this indicative work. Also other basic and acid dyes could be used.

(2) Bovarnick, M. J., The Formation of "extracellular d(-)oculose acid salt solution from cell walls of". Journal of Biology and Genetics, 1B: 17-25 (1932)

(3) Brown, Rachel, Chemical and Enzymological studies of the Inorganicus. Journal of Chemistry, 12: 5-5 (1952)


   (a) p. 16
   (b) p. 19
   (c) p. 105
   (d) p. 113

   (a) p. 27
   (b) p. 33


(11) Kooikerhoile, J. C., Studies on Capsule Formation; I Conditions under which Leptotilla cirrhata Forms a Capsule. Journal of Bacteriology, 3: 37-105 (1939)


(14) Leifson, E., and Clark, E. B., Dye Adsorption by Bacteria at varying II-ion concentrations. Stain Technology, 10: 95-109 (1941)


(17) Seastone, G. V., Capsules in young cultures of Streptococci bovis. Journal of Bacteriology, 20: 301-307 (1933)


(20) Tolstovekov, A. V., Detailed Differentiation of Bacteria by means of a mixture of gold and silver ions at different dilutions. Stain Technology, 17: 31-39 (1927)

(21) Wadsworth, Augustus, Studies on single cell Differential Methods of Staining observed on stained cocci in smear and section. Journal of Infectious Diseases, 2: 815-818 (1909)