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An Evaluation of Bacterial Flagella Staining Procedures

Dan Dalan

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An Evaluation of
Bacterial Flagella Staining Procedures

by

Dan Dalan

Bachelor of Science
University of North Dakota, 1979

A Thesis
Submitted to the Graduate Faculty
of the
University of North Dakota
in partial fulfillment of the requirements
for the degree of
Master of Science

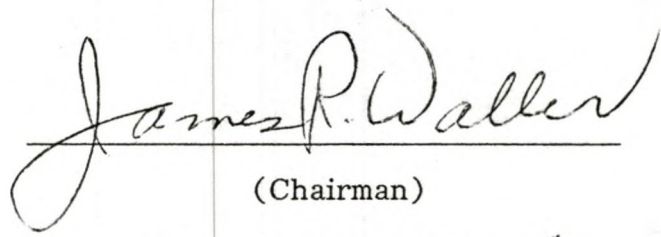
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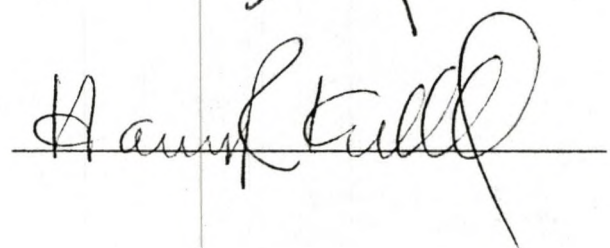
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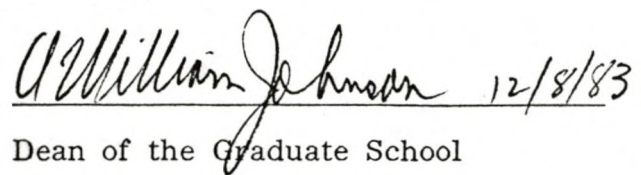


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ABSTRACT

Five currently used flagella stains and staining procedures have been studied and evaluated. A staining method that is simplest, most reliable, and highly reproducible, especially for beginning students and technicians has been assembled and recommended.

Four important variables necessary for successful flagella staining were stressed. These variables are: growth of organisms, smear preparation, staining procedure, and reagent stability.

Two important procedures which are part of some, but not all of the flagella staining methods studied are strongly recommended. One is the practice of enclosing a smear with wax pencil and adding a known, constant volume of dye. An effective ratio of staining surface area to volume of dye used is obtained with this procedure resulting in a standardization that greatly diminishes overstaining and understaining reactions. The second procedure is the use of a freshly mixed dye mordant staining mixture. This procedure is the key to reproducible production of good flagella stains that have clear backgrounds and few interfering artifacts. Dye and mordant reagents may be stored separately for long periods of time, probably indefinitely, and still provide good, clean, reproducible flagella stains when mixed just prior to use.

INTRODUCTION

Bacterial flagella are long, thin, appendages that provide bacteria with a means of locomotion. On the average, the widths are measured in the tens of nanometers and are below the resolving power of the compound light microscope. Fine structures of bacterial flagella are different from flagella of motile cells of higher plants and animals. The simpler bacterial flagella do not contain the nine plus two peripheral and axial pairs of fibrils characteristic of the flagella/cilia of eucaryotic cells. Bacterial flagella contain three fibrils that are coiled such that an overlapping arrangement mimics a compound helix of several strands (1). In fixed smear preparations these structures appear as sinusoidal curves whose wavelength is characteristic of the species (1). Chemically, bacterial flagella often consist solely of protein. The protein termed flagellin has a low molecular weight of 40,000 and usually contains only about 14 or 15 amino acids with no detectable histidine, tryptophan, hydroxyproline, cysteine, or cystine (2). The flagellin polypeptide has been shown by x-ray diffraction to display two molecular forms, that of an alpha-keratin configuration in the normal form and a cross-beta-reflection in the contracted state (2).

Studies of bacterial flagella utilize several techniques such as electron microscopy, x-ray diffraction, and light microscopy using special staining techniques such as dye-mordants and immunochemicals. Depending on the purpose and availability, one or more are utilized. For taxonomic classification, the more accurate and reliable techniques

in electron microscopy are needed and recommended. The methods of preparing bacterial cells for electron microscopy minimize detachment and damage to flagella thereby making the elucidation of flagella number and characteristics more accurate and reliable (3,4,5). In the clinical microbiology laboratory, special flagella staining techniques and a light microscope are adequate to determine the presence and arrangement of flagella for identification purposes (2,6,7,8,9).

Because flagella are too thin to be seen with the optical microscope, the width is artificially enlarged. Alcoholic dye and or aqueous silver nitrate solutions are used to stain the cells and flagella. Mordants, including tannic acid and other precipitating reagents enhance the staining reaction. During the staining reaction, the mordant is precipitated out of solution by allowing the solvent to evaporate. Precipitated solids then settle on top of the flagella and artificially enlarge the structures.

The presence of flagella, their arrangement, wavelength, and number per cell aid in the systematic classification and identification of bacteria. Flagella staining can be a useful tool in the clinical microbiology laboratory, although its use is largely restricted to reference laboratories. In general, flagella staining is used more often in taxonomic work (8), and in teaching laboratories.

A number of bacterial genera and species often encountered in the clinical laboratory require the knowledge of flagella presence and characteristics for identification. These include some fermentative, many non-fermentative gram negative rods (7), miscellaneous aerobic gram negative rods (10), and motile gram negative anaerobes from human

clinical material (11). Processing of these anaerobes has been reported to be increasing by the Centers for Disease Control Anaerobic Reference Laboratory (11). Flagella staining is then an advocated and important tool in a clinical laboratory.

There are many methods reported in the literature for staining flagella, each very similar in that properly flagellated cells are grown and a smear is prepared, mordanted, and stained. Differences lie in the reagents used, storage of staining solutions, and smear preparation.

The most often cited procedure is Leifson's flagella stain (12,13,14, 15). Another proven effective method is a silver staining technique (2,16,17). Fontana's (18) silver stain for spirochetes has been modified by Rhodes (16), and West (17), to specifically stain bacterial flagella.

The main drawback of early flagella staining methods is the variability in the results obtained. Knowledge and years of practice with flagella staining have improved the technique. There are many reports of good results from recent and currently used flagella stains (11,17,19, 20,21). However, drawbacks in reagent stability have been experienced in this laboratory and similarly listed by other authors (5,11,17,19).

The trend in modifications has been to shorten and simplify the process. Most recent attempts center on the stability of the combined dye-plus-mordant reagents.

The purpose of this study was to evaluate several current and popular flagella staining methods and recommend the most effective method, and/or assemble a method that is simple, reliable, and highly reproducible, especially for beginning students, or technicians who occasionally must perform a flagella stain.

Five current flagella staining procedures and flagella staining reagents found in the literature within the last five decades are compared in Appendices A-F. These procedures were studied and evaluated focusing on four important variables: proper growth conditions, smear preparation, staining, and reagent stability. Various authors differed in their approach to each of these variables.

HISTORICAL REVIEW

Flagella were known to many early investigators. Ehrenberg (22,23), in 1838 and Cohn (22,24) in 1872, gave early documentation of the existence of these appendages. Many formulae for staining flagella have been recommended since, although unsatisfactory results often occurred (25).

Koch (22,26) serendipitously was one of the first to pictorially document bacterial flagella. In 1877 (27), he reported improved methods of studying bacteria through staining and many technical procedures. He used logwood extract (hematoxylin) and chromic acid to stain certain bacteria, and flagella were visible in early photographs (22,26).

A staining method specifically for flagella was introduced in 1888 by Loeffler (28), using ferrous sulfate, tannic acid, and aniline dyes. The dyes and mordants used by Loeffler are the main ingredients used in flagella stains developed subsequently by other investigators. Subsequent flagella stains differ only in techniques and reagent combinations.

The flagella staining procedure has variable results and has been reported as a source of frustration in teaching laboratories (5).

Flagella staining prior to 1926 was reviewed by Wright (25). He attributed unsatisfactory and variable results to lack of knowledge of the basic factors involved. An important unexplained observation noted by Wright was the rapid chemical and physical deterioration of many mordants prepared in alcoholic solutions. The effectiveness of the staining solutions eventually diminished. No specific component of the

mordant was implicated and the deterioration could not be explained. The need for stable mordants was stressed.

All procedures for flagella staining reviewed by Wright (25) were essentially the same. Bacterial cells are grown, suspensions in water are made, a film of bacteria is smeared onto clean slides, mordanted and stained.

Methods of growing bacteria for flagella staining and several technical procedures have been simplified and standardized over the last decade, see Appendices C-F.

Several authors have recommended that cultures be grown from eighteen to twenty-four hours (11,15,17,19,20). Use of young cells is known to provide well flagellated cells with definite motility. Cultures in the logarithmic phase of active growth are recommended because flagella have a limited life span (2).

Nutrient agar or other media which sustained growth generally were used for growing bacteria. Various nutrient media, liquid or solid are used currently. Genetic variation such as loss of flagellation and motility of known motile cultures led to the use of liquid or "sloppy" semi-solid agar (2). Growth in this type of medium was reported to encourage flagellation of motile cells. Leifson (14,15), further suggested the use of carbohydrate free media. Growth in a liquid nutrient medium requires washing because excess nutrient materials such as proteins interfere with staining reactions (14,15).

The most convenient medium for growing organisms for flagella staining varies according to laboratory preference. West et al. (17), found that growth from semi-solid motility medium or trypticase soy

agar slants incubated at 25 C for eighteen to twenty-four hours was most effective. Clark (20), showed that growth on a solid medium, such as blood agar produced good smears effective for flagella staining and was less troublesome than liquid and semi-solid media.

Early methods of preparing bacteria for staining after adequate growth entailed a dilution of bacteria in distilled water to give a slightly turbid suspension, see Appendix D. Gray (29), suspended cells from a solid medium in sterile distilled water in a watch glass for twenty minutes at room temperature. Motile cells apparently rid themselves adhering of slime and medium material. From these suspensions, several loopfuls were smeared onto scrupulously cleaned slides. Thorough cleaning of slides in acid dichromate or acid alcohol and flaming has been recommended by several authors (14,20). Heat fixing, which damages flagella, was eliminated in 1902 (29), and air drying has been widely used since. West et al. (17), showed that the initial dilution step was unnecessary. Time for washing and centrifugation was eliminated. They found that the dilution obtained by adding a small quantity of bacteria from the inoculating needle to several loopfuls of sterile distilled water on any slide well cleaned by the manufacturer produced satisfactory smears and gave good flagella stains. Kodaka et al. (11), further refined the smear preparation techniques. They stressed that mixing of the bacteria in the drops of water was unnecessary and undesirable. Growth from a solid medium was picked carefully with an inoculating needle and transferred to two drops of water on a pre-cleaned slide by touching the surface of each drop of water with this bacterial mass. They claimed this avoided excessive loss of flagella.

During the early staining processes, the prepared smears generally had been mordanted first with precipitating reagents, then stained with a dye solution (31). Ryu (31) combined the dye and mordant into one solution and this combination enabled flagella staining to become a one step process.

The silver staining technique is a two step process. Silver impregnation after mordanting has been proven effective (32,33,34) and two modifications (16,17) of Fontana's (18) silver stain currently are popular flagella stains.

Successful biological stains which have satisfied most of the bacteriologist's needs in flagella staining consist of the colored organic dyes methylene blue, crystal violet, or basic fuchsin in alcoholic solutions, and inorganic silver or ferric ions in aqueous solutions (35).

All mordant solutions in flagella staining contain tannic acid, see Appendices A and B. Leifson (14) has reported that the tannic acid concentration of the mordant may vary without appreciable effect on the staining. Aqueous solutions of aluminum potassium sulfate and aluminum ammonium sulfate are added in other flagella stains to increase the amount of precipitation reactions. Ryu (31) has reported that aluminum potassium sulfate gave better results than any other salt of aluminum.

Early investigators such as Wright (25), Gray (29), and O'Toole (36), reported unstable mordants. Leifson (14), in 1951, has reported that mordant instability lies mainly in the chemical change which occurs in tannic acid in the presence of alcohol. Leifson (14) and Clark (20) claim that their combined alcoholic fuchsin and tannic acid solutions are stable indefinitely if frozen, although they acknowledge a deterioration

in the solution after several days or weeks, when stored at room temperature or refrigerator temperature, respectively.

Different strategies for storing reagents have arisen in the different modifications of flagella staining procedures. The need for a stable mordant has been known, and recent trends have been to stabilize the mordant solution, see Appendix F. Leifson (14) suggested storing the aqueous tannic acid solution separate from the alcoholic dye solution to prevent mordant deterioration. Clark (20) suggested freezing the combined dye and mordant solutions. Forbes (19) used dimethyl sulfoxide in the mixed solutions to increase stability up to three months. Kodaka et al. (11) claimed that the combined Ryu staining mixture, which combines tannic acid in alcohol, is stable indefinitely at ambient temperature. They recommend storage of the stain in plastic washing bottles for faster and more convenient procedures.

MATERIALS AND METHODS

ORGANISMS AND CULTURE MEDIUM

Proteus vulgaris from a microbiology teaching laboratory stock culture identified in this study by the API 20 E System (Analytab Products, Inc., Plainview, N.Y.) was grown for twenty four hours on blood agar plates at room temperature. The ingredients of the medium were:

	<u>gram/liter</u>
Tryptic soy agar, pH 7.2 (Difco Laboratories, Detroit, Michigan)	40
Bacto-soytone	5
Bacto-tryptone	15
NaCl	5
Bacto-agar	15
Defibrinated Sheep Red Blood Cells (Colorado Serum Company, Denver, Colorado)	50 ml

SMEAR PREPARATION

The leading edge of growth was picked using a sterilized inoculating needle. Care was taken not to pick up agar which would stain and interfere with visualization of the flagella. The needle was lightly touched to the center of each of two drops of distilled water on a new, commercially pre-cleaned microscope slide. Mixing was avoided. Previously used slides were not used in this study, but can be used if cleaned with acid alcohol (3% concentrated HCL in 95% ethanol), or chromate-sulfuric acid cleaning solution. The smear preparation was air dried at ambient temperature. A smear of proper cell density should be slightly opalescent to nearly invisible.

STANDARD STAINING PROCEDURES

The bottom two-thirds, an area 2.5 x 4.5 cm, of the slide containing the smear was enclosed with wax pencil. One milliliter of mixed staining solution (dye plus mordant) was added and allowed to act according to the recommended time for each specific staining procedure, see Appendix D.

The Ryu stain was allowed to act on the smears for various times between thirty seconds and five minutes. A staining time of one and two minutes was used for the Forbes stain. The optimum staining time for each batch of freshly prepared staining solution or stored frozen and thawed Clark and Leifson stains were determined by staining three or more different trial smears at different times, usually between five and fifteen minutes.

The West procedure differed from the other staining procedures. The mordant was added first, separate from the stain. One milliliter of solution I, the mordant, was added to cover an enclosed smear for four minutes. The slide was then rinsed with distilled water and covered with one milliliter of solution II, the staining solution. Heat was applied just until steam formed. The smear was then allowed to remain in contact with the staining solution for four more minutes without further heating.

VARIATIONS IN STAINING PROCEDURES

Staining times less than and greater than the optimum periods of staining time were determined for each staining system tested. Freshly prepared and mixed dye-mordant staining solutions, dye and mordant solutions aged separately and mixed just before use, and dye-mordant

solutions aged as a mixture were tested for speed, quality and reproducibility of flagella staining.

The area enclosing the smear and the volume of staining solution used which define the surface area to volume ratio, was varied. An enclosure of 2.5 x 4.5 cm, 2.5 x 3.0 cm, and no enclosure resulting in a 2.5 x 7.5 cm area, were stained using one milliliter of stain. In another experiment, the enclosure was kept constant at 2.5 x 4.5 cm, and 0.25 ml, 0.5 ml, 0.75 ml, and 1.0 ml volumes of stain were used.

The effect of temperature on the speed and quality of the staining reaction was determined at 4 C, 25 C, and 35 C. The stains were allowed to act on the smears inside a refrigerator, at ambient laboratory atmosphere, and inside an incubator. Differences in optimum staining time for the Clark stain were determined at each of these temperatures.

EVALUATION OF FLAGELLA STAINS

Flagella staining quality has been rated by a numbering system used by West et al. (17), which consists of the following:

1. bacteria, but no flagella visible.
2. few flagellated forms visible and flagella morphology poor.
3. few flagellated forms visible, but flagella morphology good.
4. many flagellated forms with good morphology visible, but only on isolated portions of the smear.
5. many flagellated forms with good morphology visible over the entire smear.

This 5 point system rates mainly the smear preparation quality and the definition of flagella morphology is not specific. Variations from good and bad staining and in number of flagellated forms per microscopic field always occur in different areas throughout the smear because of

varying thickness of cells produced during the smear preparation. Poor flagella morphology can be caused by understaining or overstaining.

I modified the West system to a 4 point rating system and used this new system to rate the quality of flagella stains. The rating of 4 and 5 were combined, and the ratings of 2, 3, and 5 were modified.

The new 4 point rating system was as follows:

1. bacteria, but no flagella visible.
2. few flagellated forms visible and stained flagella on most of the organisms is faint and understained.
3. few flagellated forms visible, and flagella on most of the organisms is obliterated and overstained.
4. many flagellated forms with good morphology visible over the entire periphery of the smear.

PHOTOMICROGRAPHS

A Leitz Ortholux microscope with an Orthomat microscope camera (Ernst Leitz, Wetzlar, Germany) was used to take photomicrographs at 1000x magnification. The film used was Kodak Professional ASA 200 (daylight) (Eastman Kodak Co., Rochester, N.Y.).

REAGENTS USED FOR FLAGELLA STAINS

Tannic acid (Mallinckrodt Chemical Works, St. Louis, Mo.)

Aluminum potassium sulfate (MCB Reagents, Cinn. Ohio)

Aluminum ammonium sulfate (MCB Reagents, Cinn. Ohio)

Ferric chloride (J.T. Baker Chemical Co., Phillipsburg, N.J.)

Silver nitrate (Mallinckrodt Chemical Works, St. Louis, Mo.)

Concentrated ammonium hydroxide (J.T. Baker Chemical Co., Phillipsburg, N.J.)

Crystal violet (Difco Laboratories, Detroit, Michigan)

Basic fuchsin (MCB Reagents, Cinn. Ohio)
(certified for flagella stain)

Acid fuchsin (MCB Reagents, Cinn. Ohio)
(certified for flagella stain)

NaCl (Matheson Coleman and Bell, Norwood, Ohio)

Glycerol (J.T. Baker Chemical Co., Phillipsburg, N.J.)

Trizma 7.6 (Sigma Chemical Co., St. Louis, Mo.)

Phenol (Fisher Scientific Co., Fairlawn, N.J.)

95% ethanol

Dimethyl Sulfoxide (J.T. Baker Chemical Co., Phillipsburg, N.J.)

Distilled water

PREPARATION OF WEST'S SILVER STAINING SOLUTIONS

Mix the following ingredients and store solutions I and II in different containers.

Solution I

Saturated aqueous aluminum potassium sulfate (14 gm in 100 ml distilled water)	25 ml
--	-------

10% tannic acid (10 gm in 100 ml distilled water)	50 ml
--	-------

5% ferric chloride (5 gm in 100 ml distilled water)	5 ml
--	------

Resulting mixture turns black when reagents are combined.
Store in the dark at 5 C.

Solution II

5% silver nitrate (5 gm in 100 ml distilled water)	150 ml
---	--------

Concentrated ammonium hydroxide	2 to 4 ml
------------------------------------	-----------

Slowly add concentrated ammonium hydroxide to 90 ml of 5% silver nitrate until the brown precipitate formed just redissolves. More 5% silver nitrate is added dropwise until cloudiness persists. Between

2 to 20 ml are needed. Store in the dark at 5 C. Solutions I and II are stable for at least six months.

PREPARATION OF THE RYU STAINING SOLUTION

Mix the following ingredients and store solutions I and II in different containers.

Solution I

5% phenol	10 ml
Tannic acid	2 gm
Saturated aluminum potassium sulfate (14 gm in 100 ml distilled water)	10 ml

Solution II

Saturated alcoholic crystal violet (7 gm in 100 ml 95% ethanol)	100 ml
--	--------

Mix ten parts of solution I and one part of solution II for staining. Store in dropper bottles at room temperature. Date each bottle of stain mixtures. Ryu (31) reported that the mordant solution is stable for more than seven months when kept cold. Kodaka (11), reported that the mixed dye and mordant solution is stable indefinitely.

PREPARATION OF THE CLARK STAINING SOLUTION

Mix the following ingredients:

Solution I

Basic fuchsin	3.0 gm
95% ethanol	250 ml

Dissolve and let stand overnight.

Solution II

Tannic acid	3.75 gm
NaCl	1.9 gm
Distilled water	250 ml

Mix well to dissolve. Let stand overnight.

Mix solutions I and II. Carefully adjust pH to 5.0 to 5.1 with 1N NaOH if pH is less than 5.0. Store at 4 C for 2-3 days before use for better results.

Store by freezing at -20°C, 50 ml aliquots in tubes with tight caps for future use. Date each fresh batch made.

When needed, thaw the required quantity and vortex well to remix separated water and alcohol. Store thawed stain in dropper bottles at 4 C. Put the date when the stain was thawed on the bottles. Thawed stain is stable for about a month at refrigerator temperature, for several days at room temperature.

PREPARATION OF THE FORBES STAINING SOLUTION

Add the following to screw-capped 16 x 125 mm tubes.

Acid fuchsin	0.2 gm
Basic fuchsin	0.4 gm
Tannic acid	0.2 gm
Aluminum ammonium sulfate	0.5 gm

Cap tightly, mix, and store at room temperature.

Combine the following into each tube just before staining.

95% ethanol	2 ml
Glycerol	0.5 ml
Tris buffer (Trizma 0.05M, pH 7.6)	7.5 ml
Dimethyl Sulfoxide	0.5 ml

Vortex combined solute and solvent vigorously for 3-5 minutes and then centrifuge between 900-1000 x g for two minutes.

Allow to stand at room temperature for thirty minutes before use.

Store in clean screw capped tube covered with mineral oil at room temperature for up to two weeks. Do not refrigerate or freeze. The author claims that the inclusion of 0.5 ml of dimethyl sulfoxide in the solvent solution results in a more stable stain which can be stored at room temperature or frozen at -20 C for at least three months.

PREPARATION OF THE LEIFSON STAINING SOLUTION

Mix the following solutions and store in different containers.

Solution I

NaCl	1.5 gm
Distilled water	100 ml

Solution II

Basic fuchsin	1.2 gm
95% ethanol	100 ml

Dissolve stain into alcohol and allow twenty-four hours for complete solution.

Solution III

Tannic acid	3.0 gm
Distilled water	100 ml
25% phenol	10 ml

Working stain solution is prepared by mixing equal volumes of solutions I, II, and III and stored in tightly stoppered bottles. Stain is ready for immediate use. Date each batch mixed. Author claims mixed staining solutions are stable for days at room temperature, for one to two months in the refrigerator (5 C), and months to years frozen at -10 to -20 C. Individual stock reagents, solutions I, II, and III are quite stable (14).

RESULTS

The quality ratings of staining when each of the five flagella stains was allowed to act on a smear for various periods of time are listed in Table 1. The optimum staining time which produced good results using freshly mixed reagents was shortest, one half to one minute, with the Forbes, Ryu, and West stains. The Leifson and Clark stains were effective after 12 to 18 minutes of staining. The range of staining times which produced the best quality flagella stains using freshly mixed reagents for each of the five stains served as controls for comparison of optimum staining times for aged staining mixtures and reagents.

Table 1. Quality ratings and ranges of staining time for five flagella stains.

<u>Flagella stain</u>	<u>Rating of 2. Understained</u>	<u>Rating of 4. Best quality</u>	<u>Rating of 3. Overstained</u>
Clark	<12 min	12-18 min	>18 min
Forbes	<1 min	1-8 min	>8 min
Leifson	<12 min	12-18 min	>18 min
Ryu	<30 sec	30 sec-5 min	>5 min
West	<1 min	1-40 min	a

^aThe West stain did not produce overstained smears even after 40 minutes. The total staining time of the West two-step staining procedure is presented. Half of the time is used for mordanting, the other half for staining.

When smear preparations were deliberately understained, flagella appeared faint, barely visible, or invisible, rating a 2. Overstained preparations are rated a 3 because flagella were obliterated with excess dye-precipitate complex. In addition, artifacts accumulated during over-

staining which interfered with visualization and study of flagella characteristics.

Overstaining occurred at different times for each dye-mordant system. The data in Table 2 show the quality ratings of smears when staining area and volume of stain used were varied using the Clark stain. Table 3 contains the different surface area to volume ratios for each variation in surface area and volume used in Table 2. A surface area to volume ratio of 19 and less produced a stain quality rating of 4. Surface area to volume ratios of 23 or greater produced preparations overstained to the point where even the cells and the smear as well as the flagella were obliterated by the stain.

The effects of temperature variation on optimum staining time based on quality rating of smears are presented in Table 4. A temperature of 35 C (incubator) decreased the optimum staining time from twelve minutes to ten minutes. Smears were overstained in twenty minutes at 25 C and 35 C temperatures. There was no overstaining even after twenty minutes at 5 C (refrigerator temperature) and flagella stains were still of good quality.

The effects of aging on staining reagents as judged by the quality of flagella staining using known optimum staining times are listed in Table 5. Freshly mixed stock reagents of dye and mordant solutions, whether recently prepared, or stored after five months, were equally effective in staining flagella.

Except for the Ryu staining mixture, three month old dye/mordant mixtures did not produce effective flagella stains when optimum staining times for the freshly made mixtures were used. In fact, longer staining

Table 2. Effect of variations in surface area to dye volume ratio on staining quality using the Clark stain.

Area	Volume (ml)			
	<u>0.25</u>	<u>0.50</u>	<u>0.75</u>	<u>1.0</u>
7.5 x 2.5 cm	3 ^a	3	3	4 ^b
4.5 x 2.5 cm	3	3	4	4
3.0 x 2.5 cm	3	4	4	4

^a₃ = overstained

^b₄ = best quality.

Table 3. Ratios of surface area enclosing a smear to volume of stain used.

Area	Volume (ml)			
	<u>0.25</u>	<u>0.50</u>	<u>0.75</u>	<u>1.0</u>
7.5 x 2.5 cm	75	38	25	19
4.5 x 2.5 cm	45	23	15	11
3.0 x 2.5 cm	30	15	10	8

Table 4. Effect of temperature on staining quality^a using the Clark flagella stain.

Staining Time (min)	Temperature		
	5 C	25 C	35 C
5	1	1	1
6	NT ^b	NT	1
7	NT	NT	1
8	NT	NT	2
9	NT	NT	2
10	2	2	4
11	2	2	4
12	4	4	4
13	4	4	4
14	4	4	4
15	4	4	4
18	4	4	NT
20	4	3	3

^aSee 4 point quality rating system under materials and method.

^bNT, Not tested.

Table 5. Effects of age of reagents and stain mixtures on the quality ratings^a of five flagella stains.

Flagella stain	Freshly prepared stock reagents mixed into a final staining solution just before use.	Aged dye/mordant mixtures. Stored for 3 months.	Aged stock reagents stored separately & mixed into a final staining solution just before use. Stored for 5 months.
Clark	4	2 ^b	4
Forbes	4	1	4
Leifson	4	2	4
Ryu	4	4	4
West	4	c	4

^aStaining times producing the "best quality" rating of 4 (4 point rating system) in the control group were used for each respective aged flagella stain.

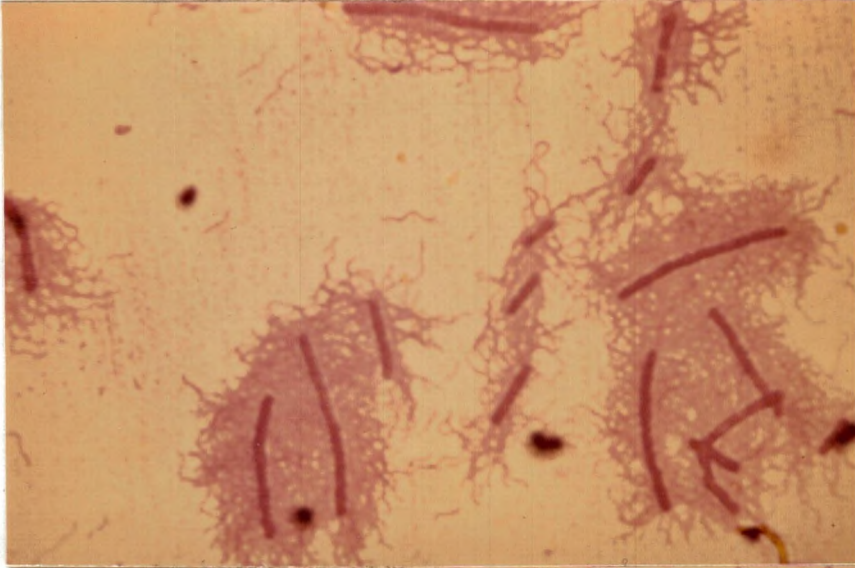
^bClark staining mixture was stored frozen and then thawed just before use.

^cWest procedure does not mix the dye and mordant for storage.

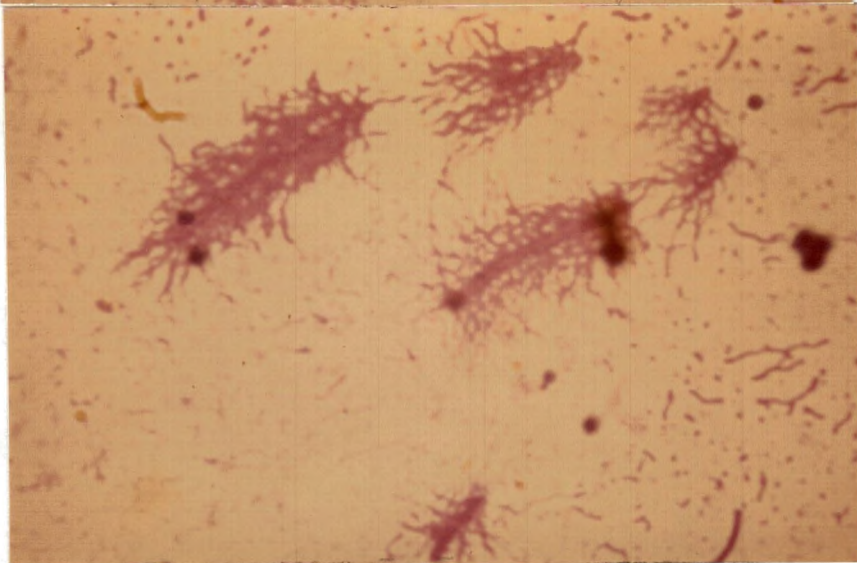
Figure 1. Bacteria and flagella stained with freshly mixed Ryu stain for A) 1 minute, B) 2 minutes, and C) 4 minutes. All three rated a 4.



A



B

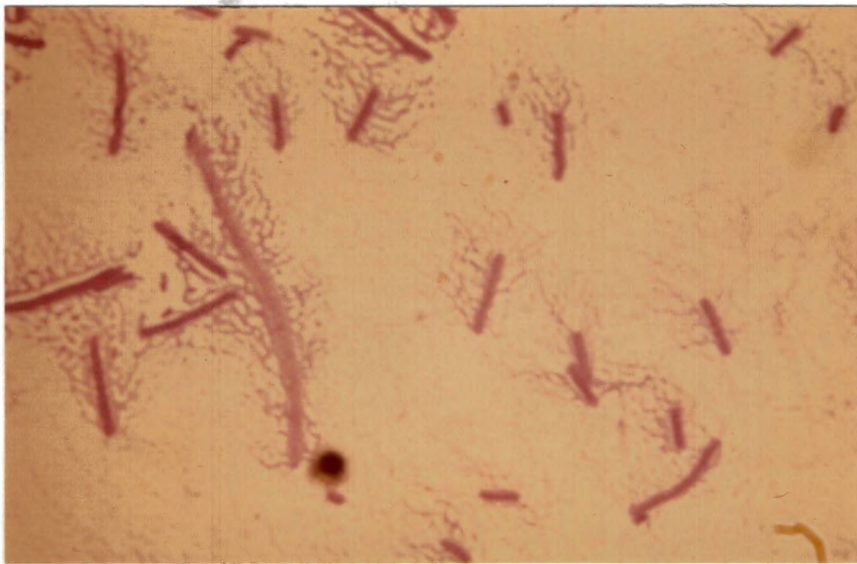


C

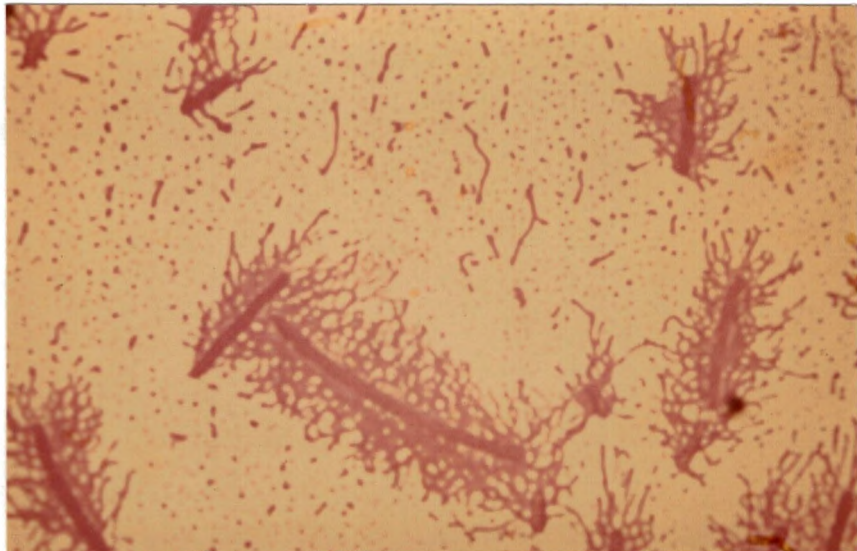
Figure 2. Bacteria and flagella stained with three month old Ryu stain for A) 1 minute, B) 2 minutes, and C) 4 minutes. Quality ratings are 2, 4, and 4, respectively.



A



B

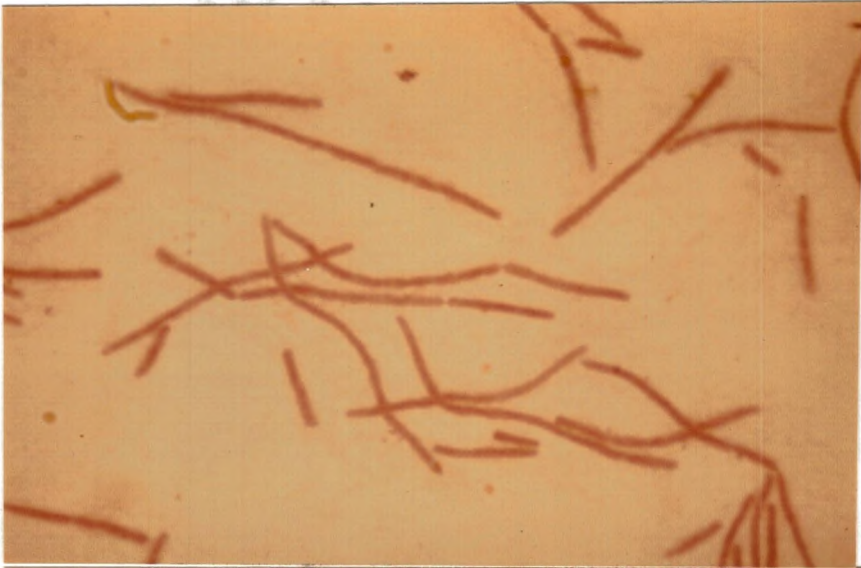


C

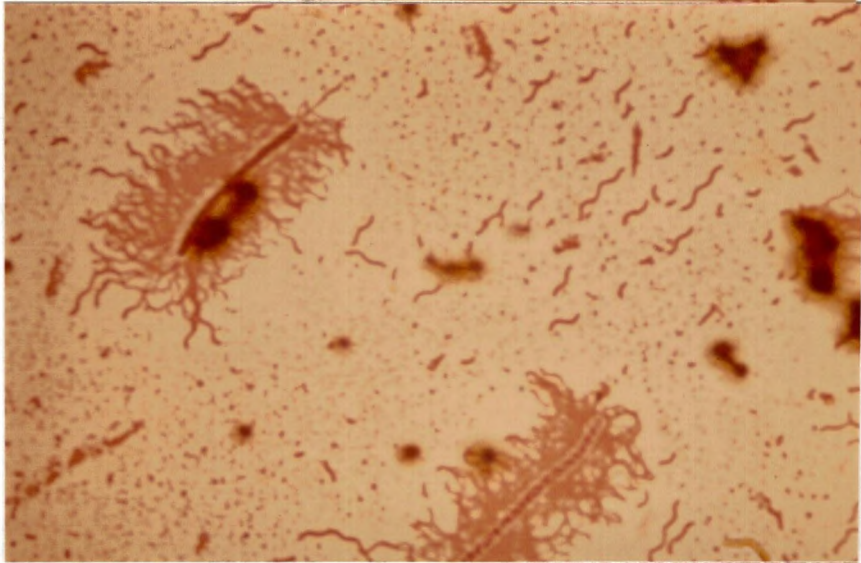
times were required to get effective flagella staining for all aged dye-mordant systems.

Figure 1 shows cells stained with the Ryu reagents for 1 min, 2 min, and 4 min. After three months, the same reagents produced the results shown in Figure 2. Longer staining times to 2 and 4 minutes were necessary to produce good flagella staining, although effective staining still occurred within a 1-5 minute range. Unpredictable optimum staining times occurred using stored frozen Clark stain. Figure 3 shows cells stained with freshly combined Clark reagents at different times. Fifteen minutes was the optimum staining time for this batch of stain. A stored, frozen aliquot of the same batch was thawed three months later. Figure 4 shows cells stained with this mixture. Note the quantity of debris on Figure 4b after 15 minutes of staining. The optimum staining time changed from 15 to more than 20 minutes. At 20 minutes staining was still light, but flagella were visible and distinguishable. However, a different bottle of stored stain from the same original batch was thawed two weeks later and produced an optimum staining time back to the original 15 minutes. In our laboratory experience, frozen Clark stain after one year required optimum staining times greater than 20 minutes. The Leifson stain is essentially the same as the Clark stain and results were very similar. The Forbes stain is most effective within days after mixing and storage of the staining mixture is not recommended. Results of this staining procedure are presented in Figure 5. The West procedure does not utilize combined dye plus mordant solutions. Results of this silver plating technique are presented in Figure 6. The photograph shows cells stained for 20 minutes.

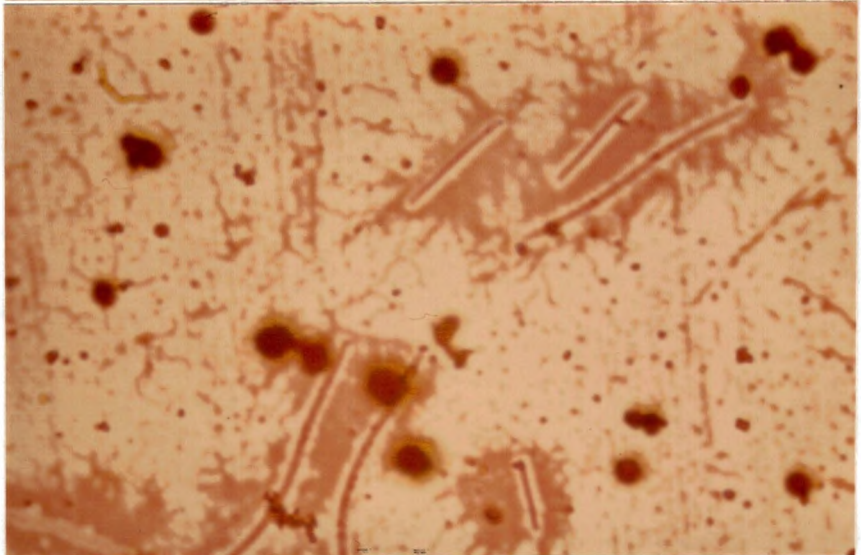
Figure 3. Bacteria and flagella stained with freshly combined Clark stain for A) 10 minutes, B) 15 minutes, and C) 20 minutes. Quality ratings are 1, 4, and 3, respectively.



A

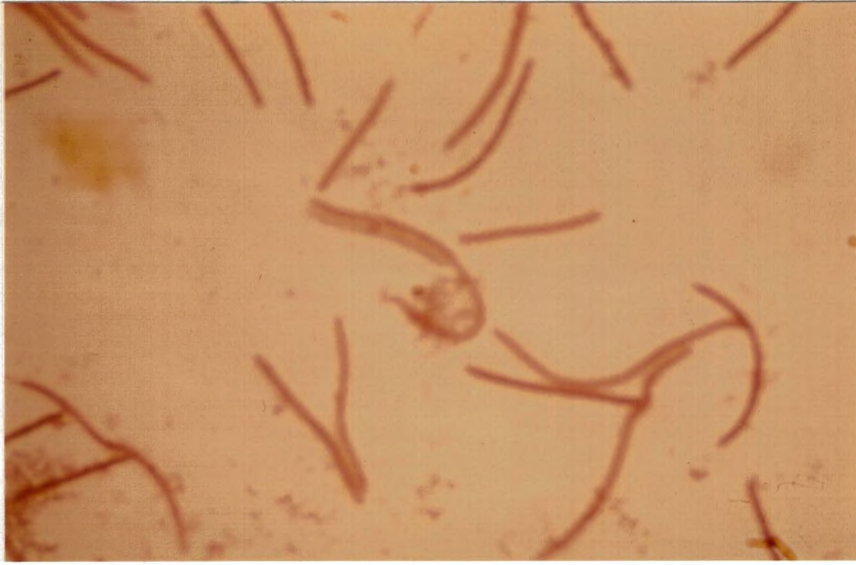


B

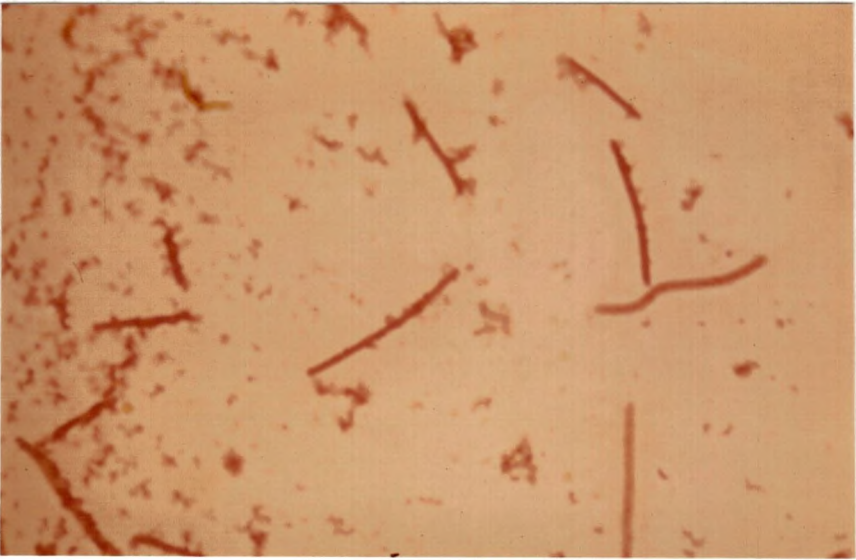


C

Figure 4. Bacteria and flagella stained with Clark reagents which was stored frozen for three months and thawed just before use. Staining times are A) 10 minutes, B) 15 minutes, and C) 20 minutes. Ratings are 1, 2, and 4, respectively.



A



B



C

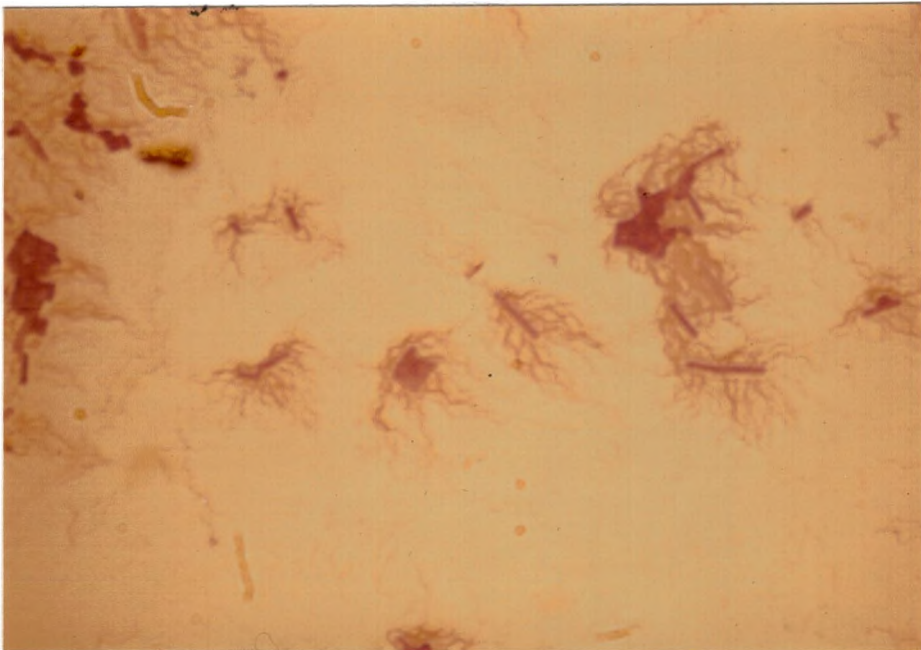


Figure 5. Bacteria and flagella stained with fresh Forbes stain for two minutes. Cells are counterstained with crystal violet. Quality rating is a 4.

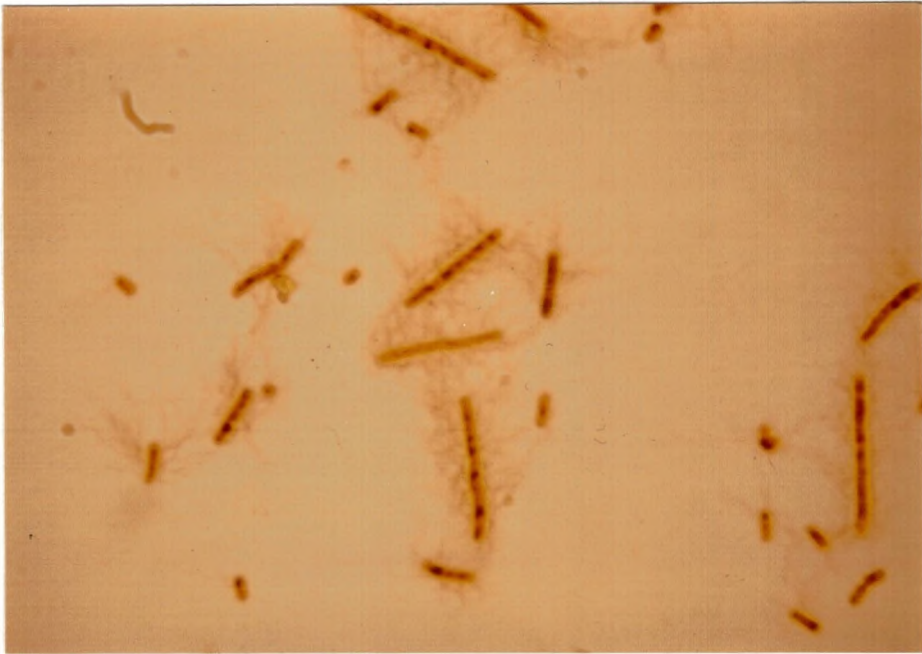


Figure 6. Bacteria and flagella stained with fresh West flagella stain for 20 minutes. Flagella appear as fine threads. Note clear background, and no flagella obliteration. Quality rating is a 4.

Normal total staining time for silver staining is 8 minutes. Eight minutes and twenty minutes staining reactions produced nearly identical results. Two factors which increase the precipitation reaction, an increase in surface area to volume ratio and an increase in staining time, did not produce overstained or obliterated flagella.

All aged dye-mordant reagents except West's produced many interfering artifacts and background staining because of the prolonged staining periods that were necessary for old mixtures.

DISCUSSION

All five flagella stains and procedures evaluated produced satisfactory results when each step in a procedure was given careful attention. Although good results can be obtained with each procedure, each flagella staining procedure has certain drawbacks, (11,17,19,20).

The major complaints about the Leifson (15) method center on the time consuming cell preparation procedures. Leifson's procedure of growing cells in liquid media requires washing and centrifuging cells before smear preparation. Scrupulously cleaned slides and flaming were required. The optimum staining time for each batch of stain mixed or thawed must be determined on trial smears before reliable results can be acquired.

Clark (20) simplified the process of growing bacteria by circumventing the use of liquid and semi-solid media. Good flagella stains can be acquired with his procedure. Reagent instability is the major drawback. Reproducible flagella stains cannot always be obtained. In our laboratory experience with the Clark stain, stored frozen batches were not always effective within the recommended 5-15 minute staining time after storage times of three months to one year. Staining times had to be increased to greater than 20 minutes which resulted in accumulation of interfering artifacts and increased background density.

The main drawback of the Forbes staining procedure (19) is the preparation of the stain. Pre-weighed stock reagents are mixed with the solvent at the time of staining, thoroughly mixed, centrifuged for two minutes and then allowed to settle for thirty minutes before the

stain can be used. The mixed stain is stable for three months at most and then only when stored under the most optimum conditions.

The West procedure (17) is a two step process. A good flagella stain can be obtained within one to forty minutes, maybe more. Overstaining, artifact accumulation, and flagella obliteration never occurred within a wide range of staining periods tested and even at conditions which caused overstaining with other staining systems. This can be explained by the slower rate of evaporation during the staining procedure of the aqueous solution containing the mordants, as compared to the alcoholic mordant solutions in the other four flagella stains. The rate of evaporation is faster with alcohol; therefore the increased amount of precipitate formed is directly related to the faster rate. This higher rate of evaporation can cause overstaining if allowed to progress. The West reagents, both dye and mordant, in aqueous solutions are stable for months. This staining procedure is a slight modification of Rhode's (16) silver plating technique. West (17) removed an aniline dye and left an ammoniacal solution of silver nitrate as the staining reagent. The major drawbacks reported with the West procedure are that it is a two step process and that the second step in staining requires heat treatment (11). After having evaluated the whole staining procedure and results, these can hardly be considered major drawbacks. This silver plating technique produced the most consistent staining with high quality ratings each time attempted. The heating step would be considered a minor drawback in the teaching laboratory. In addition, the very fine nature of the flagella produced by silver plating techniques is inadequate for demonstration.

The Ryu stain and flagella staining techniques as modified by Kodaka et al. (11), are the most recent flagella staining procedure found in the literature. The modification centers around the storage of the stain and smear preparation. Kodaka et al. (11) recommend the storage of combined dye and mordant in plastic bottles at ambient temperature so that flagella staining can be as convenient and fast as the Gram staining procedure. Few criticisms of the Kodaka staining procedure need be made. His major contributions are a refinement of the smear preparation and emphasis on use of commercially pre-cleaned slides. These procedures circumvented more time consuming procedures previously used in flagella staining. However, flagella still are easily dislodged. This should be expected because of the inherent fragility of these structures. Good flagella stains were obtained within 30 seconds and at each time attempted up to and including 5 minutes. Clearer backgrounds were obtained using freshly mixed stock reagents. Overstaining and artifact accumulation does occur, however, at staining times greater than five minutes. Kodaka's claim of indefinite stability of the mixed Ryu stain are not supported by our data. However, it was still effective after three months with only a small increase in staining time required.

The rate of evaporation is affected by ambient temperature and air circulation. The higher the rate, the greater amount of precipitate formed on the smear. Variations in temperature in a normal laboratory setting should not affect the optimum staining time. However, the high and low extreme of 35 C and 4 C does have an effect. In one experiment, the forced air from an air conditioner nearby decreased the opti-

num staining time and decreased the time which produced overstaining by five minutes. The data in Tables 2 and 3 show the results of staining when the enclosed staining area and volumes of stain used are varied. Some staining procedures call for using one milliliter of stain in areas enclosed by wax outlines, others require no enclosure, and no set amount of stain, rather, they recommend flooding the smear with the staining reagents. Flooding the smear can be relatively interpreted and addition of a few drops to several milliliters of stain may result. Because overstaining can occur when the surface area to volume ratio is too great, the use of a constant amount of stain on the smear in a standardized enclosed area of the slide is recommended. The surface area to dye volume ratio which approximates the most effective staining ratio should be used.

Reagent instability, which has been a major drawback for many of the flagella stains reviewed, is due to deterioration of the tannic acid mordant. Leifson's observation and report of tannic acid instability in alcohol suggests that any of the flagella stains in Appendix A and F that store tannic acid in alcohol should deteriorate with time and produce less effective flagella staining.

Varying stabilities of combined dye-mordant mixtures were observed in our laboratory. This can be explained by the reagent composition and storage of each of the five flagella staining reagents in Appendix A. All contain various reagents at different concentrations in the working mixture, see Appendix B. The additional precipitating reagents, aluminum potassium sulfate and aluminum ammonium sulfate increase the total precipitation reactions during flagella staining, and may contribute to

longer stability and effectiveness of the mordant-dye working mixtures that contain one or the other of these compounds. Phenol in the Ryu and Leifson stains and glycerol and dimethyl sulfoxide in the Forbes stain may in some way delay the deterioration and stabilize and preserve the effectiveness of the staining reaction for longer periods. However, all alcoholic dye-mordant mixtures containing tannic acid deteriorate in time. The strategy of separating tannic acid from alcohol for storage is best. The practice of mixing stock reagents to give a fresh working mixture for flagella staining is strongly recommended. Although a short amount of time is saved by having mixed dye-mordant solutions on hand, effective, reproducible and reliable results are not always obtained.

West's aqueous solution of tannic acid and aluminum potassium sulfate is a mordant that is stable for long periods of time. This mordant contains the same ingredients as the Ryu stain, see Appendices A and B. They differ slightly in proportions of reagent in the final staining solution. However, in the mixed Ryu stain, tannic acid is combined in alcohol, and in the West stain, tannic acid is stored as an aqueous solution. The West reagents are effective for flagella staining, but the procedures are more cumbersome than the other four flagella stains.

Again, all five flagella staining procedures and stains are adequate if directions are followed meticulously and freshly made staining mixtures are used. However, inexperienced students in teaching laboratories and technicians in laboratories where time is short, require that procedures be fast, simple and reproducible. A highly reproducible method that produces clear backgrounds and provides clear visualization

of flagella is necessary to produce dramatic and didactic effects on beginning students and reduce wasted time and error in busy laboratories. Only some of the procedures in the five flagella staining methods studied provide for these needs.

The following recommended procedures produce effective and reproducible results and are amenable to a teaching laboratory.

RECOMMENDED PROCEDURES

1. Growth of organism.

Streak pure cultures for isolation of colonies on blood agar plates. Inoculate a small area near an edge of the plate for swarming bacterial strains. Incubate at ambient room temperature for 18 to 24 hours.

2. Smear preparation.

Use new slides pre-cleaned by the manufacturer. If not available, clean slides thoroughly with soap and water, then dip into Coplin jars filled with 3% concentrated HCl in 95% ethanol for a few minutes. Rinse and air dry.

Pick cells from isolated colonies or the leading edge of growth with a sterilized inoculating needle. Do Not Touch The Agar. Lightly touch the bacterial mass to two drops of distilled water placed within a wax pencil-enclosed area of a clean microscopic slide. Do Not Touch The Slide. Do not stir or otherwise mix the bacteria into the drops. Air dry at ambient temperature. Smears should be opalescent, or nearly invisible.

Smear preparation for bacteria found in soil and aquatic environments may need special procedures (15,35,36). The smear preparation procedure recommended in this thesis, has been proven effective in

clinically important bacteria.

3. Handling of staining reagents.

Mixed stock reagents and separately stored dye and mordant solutions are stable for varying periods of time, see Appendix F. Mix the necessary quantity of dye and mordant solutions into a final staining mixture just before staining. Mixed dye-mordant solutions of the Clark, Forbes, Leifson, and Ryu flagella stains can be kept in the refrigerator for a week without loss of activity. The Ryu stain is recommended for use because, compared to the other four, the staining procedure and reagent preparation are relatively simple and rapid and the dye-mordant mixture has excellent stability at room or refrigerator temperature.

4. Staining procedures.

Enclose the bottom two-thirds of the slide, an area 2.5 x 4.5 cm, containing the smear with wax pencil. Apply one milliliter of stain to completely flood the enclosed area. Allow the Ryu stain to act for two to five minutes.

5. DO NOT POUR STAIN OFF THE SLIDE!

Flood stain off with water while the slide is at a horizontal position so excess precipitate will float off and not adhere to the smear (18).
Air dry.

6. Observe under the oil immersion objective of a microscope. Search for cells beginning at the periphery of the smear.

7. Stained smears should be preserved with Permount because cells are dislodged from the smear after repeated application of immersion oil and xylene.

CONCLUSION

There are many variables, such as reagent stability and ambient laboratory atmosphere during staining that are hard to control in flagella staining procedures. Careful individual attention to each procedures of flagella staining is required.

The Clark, Forbes, Kodaka, Leifson, Ryu, and West flagella staining methods are effective and successful if individual procedures are strictly followed; however, due to crowded conditions and the novelty of the procedures, fast, simple, and highly reproducible methods are necessary for beginning students and most technicians. Not all of these procedures studied are amenable to a teaching laboratory.

A flagella staining procedure assembled from observations made during this thesis study has been recommended. It is a composite of the procedures and techniques that are considered to be the best, simplest, most reproducible parts of the many methods studied. It should end forever, the problems previously associated with flagella staining.

SUMMARY

1. Growth of properly flagellated organisms, smear preparation, staining and reagent stability are four important variables that have to be controlled properly in order to get reproducible and successful flagella staining.
2. Effective ratios, 19 or less, of surface area enclosing the smear to volume of stain used are necessary to prevent overstaining.
3. As mixed dye-mordant solutions age, optimum staining time has to be increased which produces a thicker film of precipitate on the smears that usually obliterates flagella. Longer staining periods also produce more interfering artifacts on the smear.
4. Freshly mixed stock reagents of separately stored dye and mordant solutions provide the clearest background and the most consistent and reproducible flagella stains.
5. Growth of organisms on blood agar plates provides well flagellated cells.
6. Lightly touching a bacteria laden tip of a needle to two drops of water without mixing in an area enclosed by wax pencil, provides excellent smears with excellent retention of flagella.
7. The Ryu staining reagents produce the most reliable, most reproducible results.

APPENDICES

Appendix A

Table 6. Dye and mordant compositions and working reagents for several flagella stains.

	Dye	Mordant	Working Mixture
Ryu (1937)	Saturated crystal violet solution in 95% EtOH	10 parts saturated $\text{AlK}(\text{SO}_4)_2$ in H_2O ; 10 parts 5% phenol and 2 gm tannic acid (10%)	Mix dye and mordant 1:10
Leifson (1951)	1.2% basic fuchsin in 95% EtOH	1.5% NaCl in water. 3% tannic acid and 2.5% phenol in water. Each stored separately.	Mix dye, NaCl, tannic acid 1:1:1
Clark (1976)	1.2% basic fuchsin in 95% EtOH	1.5% tannic acid + 0.75% NaCl in distilled water	Mix dye and mordant 1:1 adjust to pH 5
West (1977)	5% AgNO_3 in distilled water plus 2-4 ml concentrated NH_4OH	25 parts saturated $\text{AlK}(\text{SO}_4)_2$ + 50 parts 10% tannic acid + 5 parts 5% FeCl	Dye and mordant, each flooded onto the slide separately at time of staining.
Forbes (1981)	2% acid fuchsin 4% basic fuchsin	2% tannic acid + 5% $\text{AlNH}_4(\text{SO}_4)_2$ in distilled water.	Dye and mordant reagents are mixed in 2 parts 95% EtOH, $\frac{1}{2}$ part glycerol, and $7\frac{1}{2}$ parts of 0.05 M Tris buffer pH 7.6. 5% (vol/vol) of dimethyl sulfoxide is optional.

Appendix B

Table 7. Proportions of mordants (wt/vol) in the mordant-dye working mixtures of five flagella stains.

	Tannic Acid	Aluminum Ammonium sulfate	Aluminum potassium sulfate
Ryu	9.1%	0	6.4%
Clark	0.75%	0	0
Leifson	1.0%	0	0
West	6.25%	0	4.4%
Forbes	2%	5%	0

Appendix C

Table 8. Methods of growing bacteria for flagella staining.

	<u>Growth of Organisms</u>
Leifson (1951)	28-48 hours. Solid, semi-solid, and broth media.
Clark (1976)	24-48 hours on blood agar plate.
West (1977)	Semisolid motility medium or tryptic soy agar slants at 25 C 18-24 hours.
Forbes (1981)	24-48 hours on both blood agar and MacConkey Agar plates.
Kodaka (1982)	17-72 hours at 35 C on blood agar plate.

Appendix D

Table 9. Methods used by several authors to prepare slides and smears for flagella staining.

Leifson (1951)	Slides scrupulously cleaned and flamed. Bacteria grown in liquid media require washing. Bacteria suspended and diluted in water. Staining area enclosed. Loopful from dilution allowed to run down tilted slide.
Clark (1976)	Slides scrupulously cleaned and flamed. Staining area enclosed. Bacteria suspended in water and a loopful allowed to run down tilted slide.
West (1977)	Commercially pre-cleaned slides. Bacteria direct from the medium suspended in 4 drops of sterile, distilled water and spread over slide surface. Staining area not enclosed.
Forbes (1981)	Commercially pre-cleaned slides. Staining area enclosed. Isolated colonies touched gently with an inoculating needle and mixed into 3 drops sterile distilled water. No visible opalescence. Spread suspension over enclosed area.
Kodaka (1982)	Commercially pre-cleaned slides. Colonies picked with a needle and touched gently to top of 2 drops of water without mixing. Staining area not enclosed.

Appendix E

Table 10. Volumes of staining reagents used on smears and length of staining time for five flagella staining procedure.

Leifson (1951)	Use one ml of stain/slide. Determine optimum time for each batch. Usually allowed to act for 10 minutes.
Clark (1976)	One ml of stain/slide. Determine optimum time for each batch. Use 5, 10, 15 minute staining times.
West (1977)	Cover smear with mordant for 4 minutes. Wash off with tap water then cover with stain, heat to steaming, remove heat source, then allow stain to act for 4 minutes.
Forbes (1981)	Use one ml of stain/slide for one minute. One ml of counterstain/slide for one minute.
Kodaka (1982)	Flood slide with stain and allow to act for 5 minutes.

Appendix F

Table 11. Dye/mordant stability of five flagella stains as claimed by authors.

Ryu (1937)	Aqueous mordant stable for greater than seven months when kept cold, 5° C.
Leifson (1951)	Stock reagents stored separately are quite stable. Mixed reagents for several weeks at 5 C, days at room temperature, months to years at -10° to -20°C.
Clark (1976)	Frozen stain stable indefinitely. Thawed stain at 20 C stable for about a month.
West (1977)	Dye and mordant stored separately, both stable at least 6 months in the dark at 5 C.
Forbes (1981)	Mixture, covered with mineral oil is stable for two weeks at room temperature or -20 C. With 0.5 gm dimethyl sulfoxide, mixture is stable at least 3 months when stored at above given temperatures. Discard after recommended storage time.
Kodaka (1982)	Ryu dye-mordant mixture stable indefinitely at ambient temperature.

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