A STUDY OF THE DETECTION OF COLIFORM ORGANISMS SEEDED IN STERILE SKIM MILK

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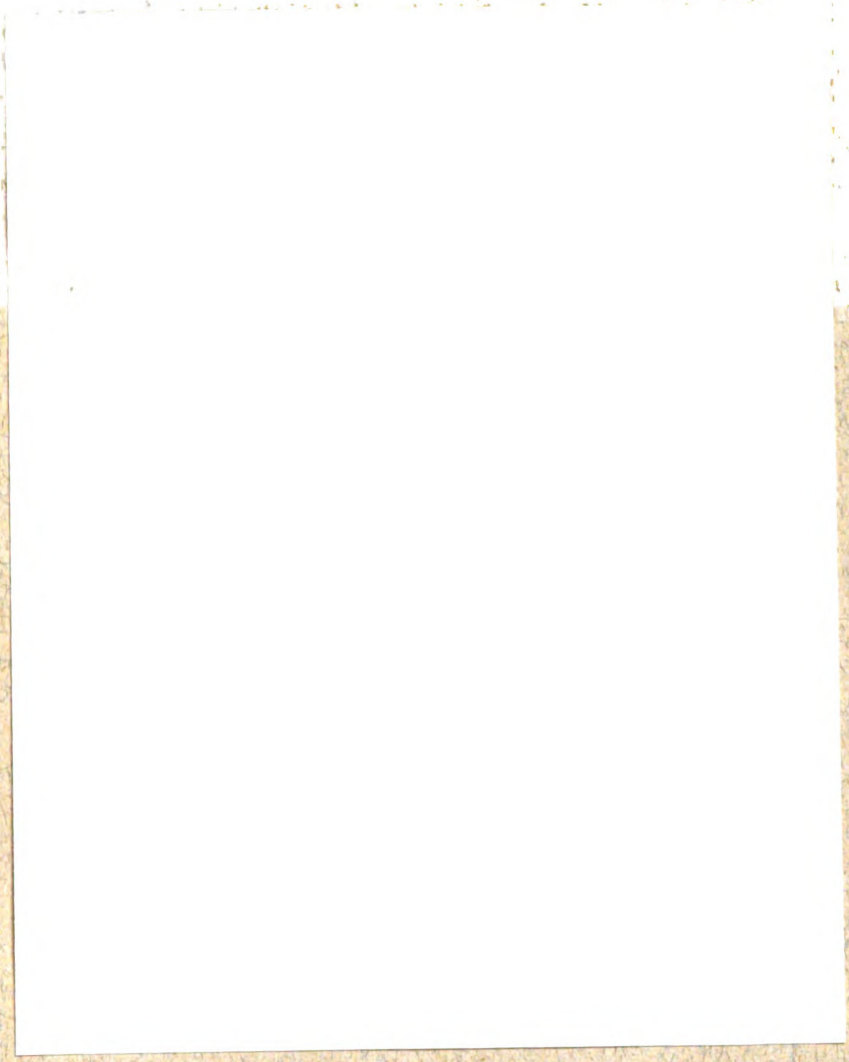

A STUDY OF THE DETECTION OF COLIFORM ORGANISMS SEEDED IN STERILE SKIM MILK

By

HANLEY MANDEL

A THESIS

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INTRODUCTION

The Breed direct microscopic examination of milk (5) has been widely used for the enumeration of bacteria in milk. Numerous efforts have been made to compare the results obtained by the direct microscopic method and those obtained by the agar plate method (5,6,14,25). Most workers have found that the two methods are not directly comparable (6,7,15,25,26). An excellent discussion of the relationship of these two procedures has been presented by Hammer (15). Duplicate samples of milk counted by these two methods have shown, in most cases, that the microscopic count is far higher than the plate count. Robertson (25) reported that the mode was 4:1 when the counts were compared, direct microscopic examination to agar plates incubated at 37°C for 48 hours. Exceptions to this relationship are to be found in data presented in the literature where the two methods have been compared (1,4,6,14,16,17,18,27). In these comparisons, numerous instances were encountered where the standard plate count was significantly higher than the count obtained by direct microscopic examination.

Baker (1), in his investigation of the direct microscopic method for counting bacteria in pasteurized milk, notes the variation in the ability of different species of bacteria to stain with methylene blue. Various stains and

staining procedures have been recommended to enhance the speed or accuracy of the direct microscopic count. The Newman-Lampert stain (25), Erb's rapid stain (12), and Shutt's rapid staining method (24) were designed to decrease the time necessary for preparation of the milk films for examination. Mallmann's acid stain (19), devised for the examination of liquid egg meats has been used for the examination of milk (8). In the examination of duplicate samples this procedure demonstrated counts higher than those obtained by the Breed technique (17). The search for better staining procedures since then has centered on the use of Mallmann's method of background decolorization.

In 1931, Whitehead (27) reported a phenomenon which is the raison d' être of this investigation. Whitehead grew various pure cultures on meat-extract peptone agar for 24 hours. The organisms were washed from the agar with sterile saline solution and seeded into milk as a cheese starter. When the milk samples so seeded were immediately examined for bacterial content, it was found that the staphylococci, streptococci and gram-positive bacilli could be easily counted by the direct microscopic method. The gram-negative coliform organisms seeded in the milk could not be detected by direct microscopic examination. These organisms could be shown to be present in large numbers by means of a subculture dilution technique employing MacConkey's broth.

Whitehead investigated this phenomenon further and found it to be consistent. In addition, the temperature of incubation was shown to affect the length of incubation time required for the organisms to become visible upon direct microscopic examination.

In this laboratory, the results obtained by Whitehead have been confirmed (3) and attempts at explanation assayed. Two main possibilities exist. The first, advanced by Baker and Peters (2), concerns the possibility of inadequacy of the staining technique employed by Whitehead and that used in this laboratory. The second, the possibility that a hitherto undiscovered life cycle of coliform bacteria may exist.

The first hypothesis is based on the known variability of stain retention by some bacteria, the difficulty with which blue-stained organisms are seen against a blue background, and the possibility that the surface charge carried by the bacteria affects the absorption of the methylene blue dye.

The second hypothesis is based on the discovery, by Mudge (2), of a life cycle of a thermophilic organism found in milk. The organism studied by Mudge had an apparent 5 to 8 minute generation time. Further studies revealed that the initial count was low because the spores failed to retain the stain and that the rapid increases in counts in

the early stages of incubation were due to the staining of the germinated spores in addition to the normal vegetative reproduction of the organism. Since the incubation of milk samples seeded with coliforms showed a similar tremendous increase in numbers when followed microscopically (5,27) during the first hour of incubation, it was reasoned that a similar cycle may be involved in this phenomenon as in that investigated by Mudge.

The purpose of this investigation, then, is to attempt to confirm the observations of Whitehead, using more rigid conditions, and to investigate the two hypotheses propounded above as a means of explanation of the failure of the direct microscopic method to detect coliform organisms seeded in milk.

The significance of this situation is readily apparent. A group of organisms of public health significance appear not to be detected by the direct microscopic examination of raw milk. Certainly the presence of numbers of coliform organisms in raw milk is equally as significant as the numbers of other saprophytic organisms found in raw milk. Moreover, the coliform organisms in raw milk may not have the same significance as those found in pasteurized milk, but may be an index of fecal contamination of the raw milk supply as well as of utensil contamination.

EXPERIMENTAL PROCEDURE

Laboratory strains of Escherichia coli, Aerobacter aerogenes, an Intermediate coliform and Micrococcus caseolyticus were checked for purity and grown on nutrient agar slopes. Incubation was at 37°C for stated periods. The culture used was washed off the agar slope with 2 ml. sterile skim milk, shaken and seeded into a tube of sterile skim milk. The dilution so made was immediately shaken and samples withdrawn for counting. Standard agar plate counts were made by the method described in Standard Methods for the Examination of Dairy Products (25). Duplicate smears were made for the direct microscopic examination by the method of Bryan, Mallmann, and Turney (6). As many slides were made as were required for the different staining procedures. The seeded milk sample was incubated at 37°C and removed at fixed intervals for the preparation of plates or milk films as described above.

The sterile skim milk was prepared from low count raw milk obtained at the Michigan State College Dairy. The skimmed milk was tubed, steamed one hour on three successive days and incubated at room temperature between treatments. After sterilization, the milk was checked for sterility by incubating and by plating. The milk prepared in this manner was free from viable organisms and from detectable dead organisms retaining the methylene blue stain.

The methylene blue stain routinely used in this laboratory is a modification of the classical Breed stain. The formulae of the methylene blue stains used in this investigation are found on page 1 of the Appendix. The pH of the Breed and Ceepryn-methylene blue stains was 6.6 to 7.0 with no adjustment.

In counting the bacteria in the milk films, 25 fields and in many cases 50 fields were counted in duplicate preparations. Where counts were exceedingly high, only 5 fields were examined. The counts reported represent the numbers of individual cells or clumps of cells retaining the stain.

Organisms, grown in nutrient broth and in sterile skim milk for different lengths of time, were also treated in like manner to determine if the phenomenon under investigation was also evident with cells grown on media other than agar. The specific conditions are detailed with each experiment under RESULTS.

RESULTS

1. Comparison of plate and direct microscopic counts of organisms seeded in sterile skim milk.

The data in Table I are the results of counting the bacteria in milk after seeding as described in the experimental procedure. The counts were made, in dupli-

cate, on samples withdrawn initially and at intervals of 30, 60, 90, and 120 minutes incubation at 37°C. No 90 minute sample of the milk seeded with M. caseolyticus was withdrawn.

The data, representing the arithmetic mean of the duplicate counts, confirm Whitehead's observations. The initial plate counts are approximately 15 to 30 times as great as the direct microscopic counts where the coliform organisms have been seeded into sterile skim milk. After 90 to 120 minutes incubation, the counts by the two methods are approximately in 1:1 ratio. The sample seeded with M. caseolyticus yielded counts in approximately a 1:1 ratio at all times. The range of experimental error between the two techniques is illustrated by the latter set of data, and is approximately 20%.

In addition, the data for the coliform organisms during the first 90 minutes in the milk demonstrate that the bacteria are not yet in the logarithmic growth phase, when followed by the agar plate method. The counts obtained by the direct microscopic method, however, would indicate that the organisms are multiplying logarithmically from the time of inoculation.

The data presented in Table II further illustrate the above results. These results were obtained in the same manner as the preceeding data. When the samples were withdrawn immediately after seeding with the coliform

organisms, the plate counts were from 15 to 100 times as high as the direct microscopic counts of each sample. After 60 minutes incubation of each sample at 37°C, the counts are in closer agreement. The data for sterile skim milk seeded with M. caseolyticus are included for comparison.

2. Comparison of direct microscopic counts when the organisms to be seeded were grown on agar slopes, in nutrient broth, and in skim milk for different periods.

The data in Table III were gathered in the same manner as the previous data, but no plate counts were obtained. The results show that when the organisms were grown in nutrient broth for 6 hours at 37°C and then seeded in sterile skim milk, the direct counts obtained immediately were not significantly lower than those obtained after the samples were incubated for 60 minutes at 37°C. The Intermediate coliform showed a 2.5-fold increase in that period, which would indicate that no lag was taking place under the conditions of transfer to the new medium.

The direct counts on milk samples inoculated from 24 and 48 hour agar slope cultures incubated at 37°C present a different picture. After 60 minutes incubation of the seeded milk samples at 37°C, there is evident a 6 to 50-fold increase in the direct counts. Such increases denote a generation time of 9 to 14 minutes. When the fact is considered that the cultures should still be in

the lag phase, it must be realized that some factor other than reproduction is involved.

The data presented in Table IV confirm these observations and the plate counts on each sample are also presented. The 6 hour broth culture has been replaced by a 5 hour broth culture, and 24 hour cultures in nutrient broth and on agar slopes compared as sources of inocula. The microscopic counts for the samples seeded with the Intermediate coliform from the 5 hour broth culture again show a large increase in the 1 hour incubation at 37°C, and this increase is paralleled in the plate counts. This observation confirms the previous postulation that this organism has no lag phase when transferred under the conditions of this experiment. Additional data, not presented in the table, when the counts were taken over shorter intervals bear out this assumption.

The bacterial counts of the milk samples seeded from 24 hour broth cultures and 24 hour agar slope cultures repeat the patterns shown before. Initially, the microscopic counts are low and the plate counts high. After 60 minutes incubation at 37°C, the microscopic counts show considerable increases, bringing them into closer correlation with the plate counts. The Intermediate coliform inoculated from a 24 hour broth culture showed one set of counts discrepant from the others. A second set obtained in like manner, did not show this discrepancy.

A 24 hour culture of E. coli grown on an agar slope at 37°C was seeded in sterile skim milk. The direct microscopic count was 42 thousand and the plate count was 1800 thousand. The sample was incubated at 37°C and 1 ml samples withdrawn after 90 minute, 3 hour and 6 hour incubation periods and seeded into tubes of sterile skim milk. The direct count on the sample seeded after 90 minutes was 172 thousand and the plate count 175 thousand. After 3 hours the direct counts on the milk sample thus seeded were 60 thousand and the plate count was 80 thousand. The sample seeded with the organisms incubated for 6 hours gave a direct count of 5400 thousand and a plate count of 4400 thousand.

It was evident that the fresh milk medium was not influencing lack of correlation between counting methods, but that the age of the organisms seeded in the skim milk determined the correlation of the direct counts to the plate counts.

The results at this point in the investigation show that coliform organisms seeded into sterile skim milk from 24 hour cultures in broth or on agar do not appear on immediate microscopical examination of the seeded sterile skim milk. After incubation for periods of 60 minutes or longer, the organisms may be found by direct microscopic examination in the numbers indicated by plate counts. The age of the cultures used for seeding and not the medium

upon which the organisms have been grown appears to decide whether the phenomenon will be demonstrated. In the remainder of this investigation, all parent cultures were grown on nutrient agar slopes for 24 hours at 37°C. These cultures are easy to handle and maintain and demonstrate the phenomenon best when seeded in the milk.

3. Microscopic appearance of the stained organisms.

The following statements hold for all the coliform strains used in this study.

The organisms, when initially seeded in the skim milk and examined after staining with the Breed stain, are extremely small and retain the stain poorly. The length of individual cells of E. coli in milk immediately after seeding from a 24 hour agar slope culture was measured, using a binocular microscope with a 1.8 mm. objective, a 10X ocular for locating the organisms, and a Leitz Wetzlar measuring vernier. The average length of the cells was 1.20 μ, with variation between the extremes of 0.32-2.06 μ. Careful examination, with 10X oculars in place of the 6X oculars used in making the counts, revealed "ghost" cells which were not stained with methylene blue.

On incubation, the cells increased considerably in length. Examination at 30 and 60 minutes after inoculation revealed larger cells in which the staining could be observed more readily. The most noticeable charac-

teristic of the poorly stained organisms was the bizarre manner in which the stain was absorbed. The cells stained as is common in the genus Corynebacterium in many instances, that is, a barred appearance was noted. In some instances, a bipolar staining was found. The larger cells, in active reproduction, retained the stain uniformly throughout their length. The irregular staining was observed only in the smaller cells present at the beginning of incubation.

The cells measured at 90 minutes had an average length of 2.52 μ, between the limits of 1.44-3.26 μ. The cells measured at 180 minutes had an average length of 3.72 μ, between the limits of 2.64-5.28 μ, Cells measured at 300 minutes averaged 1.59 μ, between the limits of 1.20-1.92 μ. The latter cells were rapidly dividing, and although small, retained the stain very well.

Little or no clumping was evident in any of the smears examined, and could not account for the low microscopic counts. No spores were encountered at any time, nor any body which might be construed to be a spore.

Because of the evidence presented to this point, namely that older cells appeared to be responsible for this phenomenon, that these "physiologically old" cells retained the stain poorly or not at all, and that no spores were found in the milk films, it was decided to discard the hypothesis that a life cycle of the coliform organisms

was involved in the phenomenon being investigated. The working hypothesis followed from this point on, was that the organisms failed to absorb the methylene blue stain to any great extent, and that the greatest numbers failed to stain at all when freshly seeded into sterile skim milk from a 24 hour agar slope culture. The remainder of the investigation therefore deals with methods designed to stain the organisms failing to stain with the Breed stain.

4. Studies of various stains and staining procedures.

As noted in the introduction, Mallmann's acid stain was the only procedure shown to stain organisms in milk which the Breed technique failed to disclose (16). Twelve replicate slides were prepared from sterile skim milk seeded with 24 hour agar slope cultures of E. coli and the Intermediate coliform. Four smears of each milk sample were prepared in this manner. All slides were dried, fixed in heat, defatted 1 minute in xylene followed by 1 minute in 95 percent ethanol. One slide was stained with the Breed stain and another with Mallmann's acid stain, counted and the results compared. The data, in the first row in Table V labeled "nil", show direct correlation. The slight increase in each count with Mallmann's stain is insignificant[1].

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[1] Significance, when used in comparing counts in this study, does not have the exact meaning as used in Statistical Analysis. In low direct counts, a difference of one bacterium between two counts may appear as a 2-fold increase. Likewise, counts of 12,000 and 36,000 must be considered as not significantly different. If the true number present were 24,000,

Recently, Dyar (10) has demonstrated the use of a cationic surface-active agent as a mordant. In addition to changing the charge of the cell wall, penetration of the cell membrane is demonstrated, and a fat dye will now stain the cytoplasm, whereas it failed to stain before treatment with the surface-active agent.

To determine whether increased counts could be obtained when the stains were used in conjunction with surface-active agents, the following procedure was performed. The remaining 10 slides, referred to above, were used. Approximate 0.01 molar aqueous solutions of alkyl dimethyl benzyl ammonium chloride, Tergitol 4, Tergitol 7, cetyl pyridinium chloride, and Duponal L-141 were prepared. Three drops of each agent were added to each smear on duplicate slides. One set of slides was then flooded with the Breed stain and the analogous set with Mallmann's acid stain. The exposure to the Breed stain was 1 minute and to Mallmann's stain 4 minutes. All slides were briefly immersed in a beaker of distilled water to remove the excess stain and air dried.

The data in Table V present the averages of 4 smears counted when stained in this manner. The significant

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[1] (cont'd)
the difference represents plus or minus one bacterium in the microscopic counts. In counts below 100,000 a 5:1 difference or greater has been taken to represent significant differences, while in higher counts a 2:1 ratio has been considered a significant difference.

results are that with the Breed stain, the cationic agent cetyl pyridinium chloride and the anionic Duponal L-141 gave approximate 10-fold increases in the count over the duplicate smears stained with the Breed stain alone. The smears treated with the cetyl pyridinium chloride were extremely clean and uniform and the background was decolorized. With the slides treated with Duponal L-141, there were likewise increased counts, but the films contained a large number of crystals and some detritus, although there were increases with other agents, the increases were less significant. When Mallmann's acid stain followed the treatment with the surface-active agents, there was considerable loss of the milk film. The film immediately floated off the slide when the stain was added in the instances where loss occurred.

The cetyl pyridinium chloride gave the best results of all the agents tested when followed by the Breed stain. It was decided to concentrate the investigation on the use of this cationic agent in conjunction with the Breed stain. To insure the use of uniform concentrations of stain and cationic agent, the two were combined and used in a coplin jar, rather than in separate solutions. The composition of the stain is given in the Appendix under the name of Ceepryn-methylene blue stain.

The concentration of the cetyl pyridinium chloride in the Ceepryn-methylene blue stain was varied. Concentrations of the agent giving final M/50, M/100, and M/200 solutions in the stain were compared with each other, with slides stained by the Breed technique, and with the standard plate count. E. coli and M. caseolyticus were the organisms seeded in the milks for these comparisons. The data summarizing these comparisons are presented in Table VI. The highest counts obtained by direct microscopic examination were in the smears stained with the Ceepryn-methylene blue stain containing M/100 cetyl pyridinium chloride. The counts are not comparable to the plate counts, but are significantly higher than the Breed count when E. coli was initially seeded in the sterile skim milk. This preparation did not affect the counts of M. caseolyticus, although the stain containing double the concentration of cetyl pyridinium chloride resulted in a general lowering of the counts.

The Ceepryn-methylene blue stain containing M/100 cationic agent in 50 percent ethanol was next compared with a like preparation where 70 percent ethanol was used in making up to volume. A 30-day old preparation and a 1-day old preparation of the regular Ceepryn-methylene blue stain was used. Direct counts using the Breed stain and standard agar plate counts were included in the comparison. The organisms seeded in the sterile

skim milk were the Intermediate coliform and A. aerogenes. The data are presented in Table VII. Both Ceepryn-methylene blue preparations containing 50 percent ethanol and M/100 cetyl pyridinium chloride gave the highest direct counts. Thirty days aging had not decreased the efficiency of the stain. Again, it must be noted that even these increased direct counts were not as high as the plate counts on the initial samples. They are however, from 5 to 6 times greater than the direct counts obtained employing the Breed stain or the Ceepryn-methylene blue stain containing 70 percent alcohol.

At this point the Ceepryn-methylene blue stain containing M/100 cetyl pyridinium chloride and 50 percent ethanol was accepted as being the best combination studied. This preparation was used from this point on.

The time of immersion of the milk films in the Ceepryn-methylene blue stain was the next factor considered. Samples of sterile skim milk were seeded with all four organisms studied and smears immediately made for direct microscopic examination. Standard agar plates were prepared at the same time. The films were fixed in heat, defatted in xylene, and rinsed in 95 percent alcohol. One slide was stained with Breed's stain for 1 minute. Three slides were stained in Ceepryn-methylene blue for

30 seconds, 1 minute and 5 minutes respectively. The slides were drained and the reverse sides gently rinsed with tap water. The counts obtained after these procedures are given in Table VII. The highest counts were obtained with the 1 minute immersion in Ceepryn-methylene blue. The plate counts were still considerably higher than these direct counts.

An interesting observation was made on the above series of smears. The extent of background decolorization appeared to be a function of the time of immersion in the Ceepryn-methylene blue stain. The longer periods of immersion resulted in greater decolorization. Immersion for 1 minute was eminently satisfactory as the background was colorless to pale blue, giving good contrast with the stained organisms. The "crest" of the film (16) retains the stain, permitting ease in focussing the microscope.

The effect of defatting the milk films before staining was assayed by examination of a parallel series of slides. The films were prepared from a sample of skim milk immediately after seeding with E. coli. One set of slides was defatted 1 minute in xylene, rinsed in 95 percent ethanol for 1 minute and stained by the Breed technique and with the Ceepryn-methylene blue stain for periods of 1 and 2 minutes. The duplicate

set was stained in identical manner, the defatting and rinsing procedures being omitted. The effect of defatting on the counts appeared to be nil. In one instance only was the count on non-defatted films higher than on defatted films (Table IX). The observation was made that the bacteria were not concentrated in the fat droplets. It had been postulated that the organisms might be present in high concentration in or on the surface of the fat globules and be lost in the defatting process. The possibility is not ruled out, as these bacteria might be inaccessible to staining and so be invisible even when present in the milk film.

In Table X, there is presented a final comparison of counts on all the organisms seeded in sterile skim milk from 24 hour agar slope cultures. Counts were made initially and after 60 minutes incubation at 37°C. The agar plate counts and direct microscopic counts where the smears have been stained by the Breed technique and the Ceepryn-methylene blue stain are compared. The direct counts using the Ceepryn-methylene blue stain are in 1:1 or 1:2 ratio to the plate counts initially and at 60 minutes, and are 5 to 30 times as great as the counts made on samples stained with the Breed stain.

The application of the staining procedure developed in this investigation to the examination of raw milk supplies was tested. Duplicate films were prepared of

40 raw milk samples submitted to this laboratory for routine examination. One set was stained with Breed's stain and the other with the Ceepryn-methylene blue stain. Fourteen samples of the 40 examined (or 35 percent) gave significantly higher counts with the new stain procedure than with the Breed stain. At no time did the Ceepryn-methylene blue stain result in significantly lower counts than those obtained with the Breed stain. The samples showing increased counts with the Ceepryn-methylene blue procedure contained rod-form organisms which were not apparent in the duplicate films stained with the Breed stain. The data are presented in Table XI.

In the course of these investigations, numerous other stains and staining procedures were attempted. None gave satisfactory results. Dyar's cell wall staining procedure (10) repeatedly dissolved the milk film from the slide. Manwell's polychrome stain (20) was exceedingly difficult to prepare, and when used at pH 7.4 dislocated the milk film on the slide. Attempts to counterstain the Ceepryn-methylene blue stain with dilute aqueous safranin proved futile. Instead of heightening the contrast between the stained organisms and the background, the contrast was diminished and the organisms were more difficult to locate.

DISCUSSION

Besides the interest in the problem of coliform organisms failing to stain with the Breed stain, another problem of considerable interest to adjacent fields arises. This problem is the means by which the surface-active cation cetyl pyridinium chloride helps the absorption of methylene blue.

In 1936 Moyer (22) showed that the electrokinetic potential of E. coli changed in various phases of the culture cycle. The electrophoretic mobility of this organism was shown to drop immediately and by the end of the lag phase to be at its lowest mobility, and, hence, lowest electrokinetic potential. The mobility remains low during the logarithmic period and then increases to its former high level during the period of negative logarithmic acceleration.

The evidence presented in this investigation on the stain absorption bears a close relation to Moyer's observations. The "physiologically old" cells seeded in the milk failed to absorb the basic methylene blue. These cells are shown by Moyer to have a high negative charge. Upon incubation, the charge drops and the now "physiologically young" cells absorb the stain.

Dyar and Ordal (11) showed that cetyl pyridinium chloride acts on E. coli to decrease the charge, reverse the charge and finally stabilize the charge at either a positive or zero potential. This was demonstrated by them with 20 to 28 hour cultures. In effect then,

if the electrokinetic potential of the cell should be the determining factor in absorption and retention of a dye, we may have by treatment with the cationic agent produced the same conditions in "physiologically old" cells as are normally found in "physiologically young" cells. The "physiologically young" cells have been demonstrated to retain the stain in this study.

The reason is not clear just why cells with a low negative or positive charge should stain with a dye like methylene blue, which itself is a cation. It would appear more likely that the more negatively charged cells should absorb the cationic dye, although the converse has been shown. The possibility exists that methylene blue may exist in a resonant structure with an induced negative pole, which would explain its absorption under these conditions. No literature on this possibility is known to the author.

The size of the cells in the course of reproduction appears to have no bearing on the stain absorption. Moyer has shown that the larger cells have greater permeability, but this appears to be a function of the reduced electrokinetic potential and not of the size per se.

The phenomenon under consideration may be explained on the basis of the change in electrokinetic potential of the cells during their culture cycle. A method for

staining these recalcitrant organisms by changing the charge has been developed and a possible mechanism of action elucidated.

It is interesting to speculate on the results of an investigation of counts on raw milk samples by direct microscopic examination employing the Breed stain and the Ceepryn-methylene blue stain, the standard agar plate count and the measurement of the coliform index by a dilution technique. Perhaps the results of such a study would lead to the adoption of a rapid microscopic method for determining the numbers of coliform organisms in raw milk samples by a comparison of direct counts obtained using the Breed stain and the Ceepryn-methylene blue stain. The coliform organisms have been shown by Dahlberg (9) to increase more rapidly than the rest of the flora in stored pasteurized milk. Because of the low storage temperatures it would be expected that a preponderance of "physiologically old" cells would be present. A technique, such as that sought for above, would be of immense value in the control of these stored milk supplies.

SUMMARY

1. These studies have shown that only a minor portion of the coliform organisms seeded in milk from 24 hour agar slope cultures grown at 37°C are detected by the conventional direct microscopic examination. These findings are in agreement with the observations of Whitehead.

2. The hypothesis of a life cycle of coliform organisms as an explanation of the phenomenon is rejected. The hypothesis that coliform organisms fail to retain the stain under these conditions is upheld.

3. A method for staining the milk films to bring the direct microscopic counts and the agar plate counts into closer correlation is developed and described.

4. The Ceepryn-methylene blue stain developed was applied to the routine examination of raw milk supplies. Of 40 samples stained in this manner, 35 percent were shown to yield significantly higher counts than with the Breed stain.

5. The theory of mode of staining is discussed in reference to the use of the cationic surface-active agent and the basic dye employed in the stain.

REFERENCES

1. Baker, M. P. 1946 The direct microscopic count as a method for counting bacteria in pasteurized milk. J. Dairy Sci., 29, 506.

2. Baker, M. P. and Peters, I. I. (Personal communication).

3. Bortree, A. L. (Unpublished data).

4. Brannon, J. H. 1940 Studies of the resazurin test for milk. Milk Plant Monthly, 29, 51-55.

5. Breed, R. S. 1911 The determination of the number of bacteria in milk by direct microscopical examination. Centralbl. f. Bakt., II Abt., 30, 337-340.

6. Brew, J. D. 1929 The comparative accuracy of the direct microscopic and agar plate methods in determining numbers of bacteria in milk. J. Dairy Sci., 12, 304-319.

7. Brew, J. D. and Breed, R. S. 1945 The bacteria "count"- an estimate capable of accurate interpretation. A. J. P. H., 35, 693-698.

8. Bryan, C. S., Wallmann, M. L. and Turney, G. J. 1944 Some microscopic technics for determining the bacteriological quality of milk. Mich. Agr. Exp. Sta., Circ. Bull. 186.

9. Dahlberg, A. C. 1946 The relationship of the growth of all bacteria and coliform bacteria in

pasteurized milk held at refigeration temperatures. J. Dairy Sci., 29, 651-655.

10. Dyar, M. T. 1947 A cell wall stain employing a cationic surface-active agent as a mordant. J. Bact., 53, 498.

11. Dyar, M. T. and Ordal, E. J. 1946 Electrokinetic studies on bacterial surfaces. I. The effects of surface-active agents on the electrophoretic mobilities of bacteria. J. Bact., 51, 149-167.

12. Erb, N. M. 1929 A rapid stain for the direct microscopic examination of milk. J. Lab. and Clin. Med., 14, 377-379.

13. Hammer, B. W. 1938 Dairy bacteriology. 2nd ed., John Wiley and Sons, Inc., New York, 1-19.

14. Jenkins, H. 1941 A comparison of microscopic and 32°C TGY agar plate counts on raw milk. J. Milk Tech., 4, 514-517.

15. Levowitz, D. 1944 Reproducible data by microscopic method. Assoc. Bull. Int. Assoc. Milk Dealers, 36th year, 139-150.

16. Mallmann, W. L. and Bryan, C. S. 1943 Emergency use of the laboratory during the war. Assoc. Bull. Int. Assoc. Milk Dealers, 35th year, 175-188.

17. Hallmann, W.L., Bryan, C.S. and Baten, W.D. 1944 A study of methods for the examination of raw milk with suggested improvements. J. Milk Tech., 7, 315-321.

18. Hallmann, W. L., Bryan, C. S. and Fox, W. P. 1941 A new microscopic procedure for the detecting and locating of the source of thermoduric organisms in milk. J. Milk Tech., 4, 195-199.

19. Hallmann, W. L. and Churchill, E. S. 1942 A rapid test for determining bacteria in liquid eggs. U. S. Egg and Poultry Magazine, 48,406-407, 427-429.

20. Manwell, R. D. 1945 The J. S. B. stain for blood parasites. J. Lab. and Clin. Med., 30, 1078-1082.

21. Moyer, L. S. 1936 Changes in the electrokinetic potential of bacteria at different phases of the culture cycle. J. Bact., 32, 433-464.

22. Mudge, C. S. 1930 A life cycle of a thermophilic organism. Proc. Soc. Exp. Biol. and Med., 28, 202-203.

23. Robertson, A. H. 1921 The relation between bacterial counts from milk as obtained by microscopic and plate counts. N. Y. State Exp. Sta., Tech. Bull. 86.

24. Shutt, D. B. 1947 A rapid staining method for the microscopical examination of milk. Stain Tech., 22, 1-2.

25. Standard methods for the examination of dairy products. 8th ed., A. P. H. A., 1941.

26. Watrous, G. H. Jr. and Doan, F. J. 1947 Observations on the use of a modified direct microscopic method for estimating bacterial quality of raw and pasteurized milk. J. Milk and Food Tech., 10, 269-276.

27. Whitehead, H. R. 1931 A note on the direct microscopic count of bacteria in milk. J. Dairy Res., 2, 80.

STAIN FORMULAE*

Modified Breed Stain:

10 ml saturated alcoholic methylene blue (certified for bacteriological use).

90 ml 50 percent ethanol.

Mallmann's acid Stain:

0.2 gm methylene blue (certified for bacteriological use)

100 ml 95 percent ethanol

1 ml concentrated hydrochloric acid

Ceepryn-methylene blue stain:

3.4 ml 10 percent aqueous cetyl pyridinium chloride

10 ml saturated alcoholic methylene blue (certified for bacteriological use)

86.6 ml 50 percent ethanol

* All staining was done in Coplin staining jars except where otherwise indicated.

RECOMMENDED STAINING PROCEDURE

1. Air dry slides in horizontal position.
2. Fix by heating in flame until too hot to hold with fingers. Allow to cool.
3. Defat 1 minute in xylene. Drain slide of xylene.
4. Rinse 1 minute in 95% ethanol. Drain slide of ethanol.
5. Immerse slide in Ceepryn-methylene blue solution. Drain excess stain from slide.
6. Wash reverse side of the slide in a gentle stream of tap water.
7. Drain and dry.

TABLE I

Comparison of plate and direct counts of bacteria in milk after periods of incubation at 37°C.

	Bacterial counts*(thousands)				
Incubated (minutes)	0	30	60	90	120
Organism					
E. coli					
plate	299	330	327	410	540
direct	9	42	60	162	435
A. aerogenes					
plate	653	1690	1310	1290	1750
direct	48	106	120	795	1250
Intermediate					
plate	292	253	565	1190	1660
direct	18	96	261	1080	1930
M. caseolyticus					
plate	700	720	620	--	690
direct	510	510	480	--	600

* Average of duplicate counts.

TABLE II

Comparison of plate counts and direct counts of bacteria seeded in sterile skim milk.

Bacterial counts (thousands)

Organism	Initial		After 60 mins.	
	Plate	Direct	Plate	Direct
A. aerogenes	375	48	550	615
	653	48	1310	120
	1350	12	--	--
	1000	36	1340	900
E. coli	4230	102	6000	6060
	299	9	327	67
	630	30	--	--
	1470	24	1560	450
	370	54	--	--
	1800	42	--	--
Intermediate	5450	165	7350	7400
	262	18	565	261
	448	36	810	210
	8250	192	21900	9450
	1870	12	--	--
	7000	72	7600	3000
N. caseolyticus	160	192	205	348
	1020	500	--	--
	700	510	620	480

TABLE III

Comparison of direct counts of milk samples, initially and after 60 minutes incubation, when seeded with cultures from different sources .

Milk seeded with	Bacterial counts* (thousands) Initial	After 60 minutes
6 hour broth		
E. coli	48	50
A. aerogenes	78	90
Intermediate	150	396
48 hour agar slope		
E. coli	6	36
A. aerogenes	15	108
Intermediate	4	132
24 hour agar slope		
E. coli	3	72
A. aerogenes	9	495
Intermediate	4	162

* Average of duplicate counts.

TABLE IV

Comparison of direct and plate counts of milk samples, initially and after 60 minutes incubation, when seeded with cultures from different sources.

Milk seeded with	Bacterial counts* (thousands)			
	Initial		After 60 minutes	
	Plate	Direct	Plate	Direct
5 hour broth				
E. coli	156	144	192	246
A. aerogenes	26	30	35	24
Intermediate**	43	150	160	252
	69	198	510	432
24 hour broth				
E. coli	145	78	160	156
A. aerogenes	110	12	226	216
Intermediate**	77	135	383	312
	712	263	1220	1170
24 hour agar slope				
E. coli	279	9	327	60
A. aerogenes	653	48	1310	120
Intermediate	262	18	565	261

* Average of duplicate counts.

** See text.

TABLE V

Comparison of direct microscopic counts when replicate slides are stained with Breed's stain and Hallmann's acid stain after treatment with surface-active agents.

Direct bacterial counts*(thousands)

Surface-active agent	E. coli		Intermediate	
	Breed	Hallmann	Breed	Hallmann
nil	6	7.5	6	7.5
alkyl dimethyl benzyl ammonium chlcride	16	**	15	**
cetyl pyridinium chloride	96	**	53	**
Tergitol 4	24	***	36	***
Tergitol 7	***	***	***	***
Duponal L-141	66	18	51	4

* Average of quadruplicate counts.

** Patchy staining, abundance of detritus and crystals.

*** Milk film washed off slide.

TABLE VI

Comparison of direct microscopic counts when the milk films have been stained with the Breed stain and the Ceepryn-methylene blue stain containing varied concentrations of cetyl pyridinium chloride. The agar plate counts are included for comparison.

Organism and stain employed*	Bacterial counts (thousands) Initial		After 60 minutes	
	Direct	Plate	Direct	Plate
E. coli				
Breed	24	1470	450	1560
Ceepryn-M.B. M/100	132		1140	
Ceepryn-M.B. M/200	60		480	
Ceepryn-M.B. M/50	12		660	
M. caseolyticus				
Breed	510	700	480	620
Ceepryn-M.B. M/100	450		480	
Ceepryn-M.B. M/200	420		500	
Ceepryn-M.B. M/50	180		72	

* Ceepryn-M.B. is the Ceepryn-methylene blue stain in 30 percent ethanol, with the indicated concentration of cetyl pyridinium chloride.

TABLE VII

Comparison of one day old and thirty day old preparations of the Ceepryn-methylene blue stain, the Ceepryn-methylene blue stain prepared with 70 percent ethanol, the Breed stain and the standard plate count.

Organism and stain employed*	Bacterial counts (thousands) Initial		After 60 mins	
	Direct	Plate	Direct	Plate
A. aerogenes		1000		1340
Breed	36		900	
Ceepryn-M.B. 30 day	180		1260	
Ceepryn-M.B. 1 day	160		1120	
Ceepryn-M.B. 70% ethanol	48		1050	
Intermediate		7000		7600
Breed	72		3000	
Ceepryn-M.B. 30 day	640		5400	
Ceepryn-M.B. 1 day	540		5800	
Ceepryn-M.B. 70% ethanol	72		5200	

* Ceepryn-M.B. is the Ceepryn-methylene blue stain containing M/100 cetyl pyridinium chloride in 30 percent ethanol except where indicated as 70 percent ethanol.

TABLE VIII

Comparison of counts obtained by direct microscopic examination when the time of immersion in Ceepryn-methylene blue stain is varied, when stained with Breed's stain and by the standard plate count.

Organism and method of examination**	Bacterial counts*(thousands)			
	E. coli	Intermediate	A. aerogenes	M. caseolyticus
Direct, stained by:				
Breed	30	12	12	500
Ceepryn-M.B. 30 sec	42	24	30	590
Ceepryn-M.B. 1 min	138	100	210	525
Ceepryn-M.B. 5 min	84	66	170	520
Plate count	630	1870	1350	615

* Average of duplicate counts made immediately after inoculation.

** The Ceepryn-methylene blue stain is the solution containing M/100 cetyl pyridinium chloride in 30 percent ethanol.

TABLE IX

Comparison of the standard plate counts and the direct microscopic counts obtained on milk films defatted and not defatted before staining by different methods. All milk samples have been seeded with *E. coli*.

Time incubated and method examined by:	Bacterial counts*(thousands) Films defatted	Films not defatted
Initial counts		
Direct		
Breed	54	30
Ceepryn-M.B. 1 min	150	150
Ceepryn-M.B. 2 min	50	140
Plate	370	370
After 90 minutes		
Direct		
Breed	265	300
Ceepryn-M.B. 1 min	320	370
Ceepryn-M.B. 2 min	315	375
Plate	380	380

* Average of duplicate counts.

TABLE X

Comparison of agar plate counts and direct counts employing the Breed and Ceepryn-methylene blue stains.

Bacterial counts* (thousands)

Milk seeded with	Initial			After 60 minutes		
	Direct		Plate	Direct		Plate
	Breed	Ceepryn-MB		Breed	Ceepryn MB	
E. coli	102	3130	4230	6060	5900	6000
Intermediate	192	4770	8250	9450	11100	21900
A. aerogenes	48	204	375	615	615	550
M. caseolyticus	192	174	160	348	270	205

* Average of duplicate counts.

TABLE XI

Comparison of direct microscopic counts of raw milk samples using the Breed stain and the Ceepryn-methylene blue stain on duplicate smears of each sample.

Direct Bacterial counts (thousands)

Counts increased using Ceepryn-M.B. stain			Counts in agreement using either stain		
#	Breed	Ceepryn-M.B.	#	Breed	Ceepryn-M.B.
1	12	84	2	12	24
5	90	750	3	12	48
7	12	660	4	120	156
11	50	156	6	12	12
15	91	288	8	12	24
18	315	600	9	90	84
21	540	990	10	500	570
23	360	900	12	12	36
24	400	750	13	200	238
25	60	240	14	50	36
28	300	1200	16	700	800
29	200	900	17	60	84
32	90	230	19	75	84
33	305	1100	20	100	144
			22	1500	1500
			26	2000	2700
			27	520	690
			30	120	150
			31	60	60
			34	60	84
			35	120	120
			36	1500	1300
			37	180	190
			38	75	84
			39	25	48
			40	80	72