ASSAYS FOR UDDER IRRITATION BASED UPON DEOXYRIBONUCLEIC ACID CONTENT OF MILK SOMATIC CELLS

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Max J. Paape

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ABSTRACT

ASSAYS FOR UDDER IRRITATION
BASED UPON DEOXYRIBONUCLEIC ACID CONTENT
OF MILK SOMATIC CELLS

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A Michigan Mastitis Test (MMT) for udder irritation was developed which produced results equivalent to the California Mastitis Test (CMT) and the Milk Quality Test (MQT). The MMT is an open formula gelation test and the ingredients can be purchased and the reagent prepared with the aid of a pan balance.

In the course of a study of milk leucocytes, the commonly used stains were found to be unsatisfactory because background staining masked the leucocytes, or because of a lack of nuclear-cytoplasmic resolution within the leucocytes. A pyronin Y - methyl green staining procedure was developed which greatly improved the microscopic resolution of milk somatic cells. Furthermore, the pyronin Y - methyl green stain resulted in an approximate 25% reduction in smear variance and in an approximate 50% reduction in count variance when compared with Wright's stain.

In view of the evidence that deoxyribonucleic acid (DNA) was the material responsible for the gelation in the CMT, MQT, and MMT reactions, the DNA-specific Feulgen

reaction was modified, and was shown to be less variable than the MQT and more closely related to leucocyte numbers than the MQT.

ASSAYS FOR UDDER IRRITATION BASED UPON DEOXYRIBONUCLEIC ACID CONTENT OF MILK SOMATIC CELLS

By

Max J. Paape

A THESIS

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BIOGRAPHICAL SKETCH

Max J. Paape was born at Port Chester, New York, on December 26, 1936. He received his elementary education in Our Lady of Mercy Parochial School, and was graduated from Port Chester Senior High School in June, 1954.

In September, 1954, he enrolled at the Long Island Agricultural and Technical Institute, Farmingdale, New York, majoring in Animal Husbandry. He was graduated in June, 1956, with the degree of Applied Arts and Science.

From September, 1956 to June 1959, he completed his undergraduate requirements leading to a Bachelor of Science in Agriculture Extension at Michigan State University.

In September, 1960, he enrolled in the graduate school of Michigan State University, Department of Dairy. He received the Master of Science degree in March, 1963.

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INTRODUCTION

The term mastitis is defined as an inflammation of the tissues in the cow's udder or teats. Many methods have been suggested for the detection of udder inflammation based on changes in the milk composition (lactose, chlorides, solids), pH, cell count, while other methods are based on the organisms involved. From the standpoint of reliability leucocyte counts are considered the most accurate method of detecting mastitis (2, 36).

Certain management practices such as the improper use of the milking machine will increase the leucocyte count of milk. If corrected in time mastitis could be prevented. One objection to most tests for mastitis is that they do not detect the condition in the early stages. The leucocyte count detects the condition much sooner than some of the other tests (2).

The purpose of the research reported in this thesis was to develop several new methods for detecting udder irritation based upon the leucocyte numbers of milk. These methods are presented as four experiments with each experiment designed to study a specific possibility of improving tests for mastitis.

EXPERIMENT I. THE MICHIGAN MASTITIS TEST

A review of the literature dealing with general mastitis tests and the gelation tests will be presented in this section of the thesis while the literature dealing specifically with the other tests for mastitis will be reviewed in the appropriate sections.

Review of Literature

Cole and Easterbrooks (13) observed a leukopenia in dairy cows with acute mastitis. Similarly, Schalm (40) and Theilen et al. (45) reported that a leucocytic response to mastitis is characterized by a rapid reduction of leucocytes in the blood as they move into the area of injury in the udder. Pattison et al. (35) found a predominance of leucocytes in the udders of goats infected with Streptococcus agalactiae. Hughes (19) showed that a high cell count was associated with inflammed quarters. McFarlane et al. (28) and Chu (12) concluded that high cell counts were an indication of mastitis. Furthermore, it has been shown by many workers, Barnum and Newbould (4), Jensen (21), Leidl and Schalm (24), Leidl et al. (25), and Schalm (41), that the California Mastitis Test (CMT) (42) and the Milk Quality Test (MQT) (34) reactions, used so widely by dairymen and by veterinarians, are indirect measures of the concentration of leucocytes in the milk.

Both the CMT and MQT have received widespread

acceptance for field diagnosis of mastitis. Albright (1) describes the use of the CMT as an effective tool in a herd management mastitis control program. In California (38, 39) the CMT is currently being applied to about 80,000 cows each month. Reports by Jackson (20), McKay (30), and Steere (43) indicate favorable results by using the CMT in mastitis control programs.

Marshall and Edmondson (29) suggested that the CMT can be of great value to the practitioner in his evaluation of the herd mastitis situation. Barnum (3) suggested that when the CMT is applied at monthly intervals it is possible to determine the infection status of each quarter. He also presented evidence to indicate that quarters infected with Streptococcus agalactiae, Staphylococcus aureus, or other organisms that produce a chronic infection, consistently show a positive CMT reaction. Furthermore, he suggested that the CMT be employed as a screening test when introducing new cows into a herd, and that a negative CMT on all quarters of a milk cow is a reasonable criterion for the animal's introduction into the milking herd.

The CMT has also been used as a quality test for milk samples collected from bulk tanks. Temple and Haller (44) have shown that CMT scores were lower on bulk milk for those herds enrolled in the New York mastitis control program. Leidl and Schalm (24) suggested using the CMT

on bulk tank samples to detect the majority of the herds which produce abnormal milk. Gray and Schalm (15) report that a reduction of bulk milk scores to negative values could lead to an increase of 14 to 20% in milk production, as well as a significant improvement in the quality of milk. Holt (18) reported that one might expect an increase in butterfat production through the proper use of the CMT.

This evidence indicates that the CMT and MQT have an important place in a mastitis control program. However, the cost of the CMT and the MQT reagents may be a deterrent to their more extensive use. A less expensive reagent may encourage a more effective mastitis control program because more dairymen would participate. The purpose of this experiment was to develop an open formula gelation test for mastitis similar in reaction to the closed formula CMT and MQT, and to present statistical evidence to determine if there was any difference in the results obtained when using these three reagents.

Materials and Methods

The open formula gelation test, hereafter called the Michigan Mastitis Test (MMT), was prepared by diluting 19.0 g of sodium alkylarylsulfonate[1], 13.5 g of sodium hydroxide[1] (analytical reagent), and 1.5 g of methylene

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[1] Available from Fisher Scientific Co., 1458 N. Lamon Ave., Chicago 51, Illinois.

blue chloride to one gallon (3,785 ml) in softened tap water. These reagents were mixed to obtain complete dissolution.

Ninety-one milk samples, each consisting of about 30 ml of foremilk, were obtained daily over a period of two weeks from Holstein cows in various stages of lactation. The samples were immediately tested in duplicate using the CMT, MQT, and MMT within 30 minutes after the milk was obtained. The CMT and MQT procedures were performed by adding 3.50 ml of reagent to 3.00 ml of milk in a paddle and scoring the degree of gelation according to the manufacturer's recommendation, except that gelation scores of 0, 1, 2, 3, and 4 were substituted for 0, T, 1, 2, and 3, respectively, to facilitate statistical analysis. The procedure employed for the MMT was identical except that the MMT solution was used in place of the CMT or MQT test reagents.

In a second trial, which was designed to determine whether or not more readily available reagents could be substituted for those in the MMT solution, 52 foremilk samples were tested in duplicate with CMT and with a Lye Mastitis Test solution, hereafter called LMT. This LMT solution was prepared by diluting 2.5 level teaspoons (13.5 g) of lye[2] and four level teaspoons (19 g) of liquid

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[2] Red Seal, high test Lyons New Flake Lye. Active ingredients: 96% NaOH, 2% Sodium Carbonate, and 2% inert ingredients.

detergent[1] in one gallon of soft tap water. After gentle mixing to dissolve the lye, the solution was allowed to stand for 24 hours to permit clearing. The clear portion was then decanted into another container with care to avoid remixing the settled out impurities, which were discarded. One level teaspoon (6 g) of navy blue dye[2] was added to the clear test solution to produce the desired color.

Results and Discussion

The average scores for 91 milk samples in the initial experiment are shown in Table 1 with the within sample standard deviations and coefficients of variation. The average scores for the CMT, MQT, and MMT do not differ significantly ($P > .50$). Although the use of the MMT resulted in lower total and relative variables, the differences among the standard deviations and among the coefficients of variation for the three tests are small relative to the average variabilities. The correlation of the average scores of CMT with MQT was 0.975; of CMT with MMT, 0.962; and of MQT with MMT 0.969. Each of these correlation coefficients were highly significant ($P < .001$).

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[1] Swan liquid detergent.

[2] Rit tints and dyes, navy blue 30.

TABLE 1

Average CMT, MQT, and MMT gelation test scores and within sample standard deviations and coefficients of variation

Test	Average gelation scores	Within Sample	
		Standard deviation	Coefficient of variation
California Mastitis Test (CMT)	1.68	0.33	20%
Milk Quality Test (MQT)	1.76	0.38	21%
Michigan Mastitis Test (MMT)	1.81	0.32	18%
Average	1.75	0.34	20%

This statistical evidence demonstrates that little difference exists among the test scores obtained from the three tests employed, and that a choice among them may be made based upon other factors such as personal preference, availability of reagents, or cost. The MMT solution may be made with a substantial reduction in cost. On the other hand, prepared MQT and CMT solutions may be more practical for those with no facilities for accurate weighing.

Since the Methylene Blue Chloride simply serves to make the milk gel more visible, other coal tar dyes may be used in its place at the discretion of the user. In fact, when the test procedure was performed in black bakelite jar lids, no color additive was necessary because the milk itself provided adequate contrast with the dark background.

In the second trial, the average test score for CMT was 1.72 which was not significantly different ($P > .50$) from the 1.77 score for LMT. The within sample standard deviations were 0.22 and 0.36, and the coefficients of variation were 13% and 20%, respectively, for the CMT and the LMT. Although these measures showed greater variability between duplicate LMT determinations, the differences were relatively small for most practical purposes. The correlation between CMT and LMT score was 0.951 ($P < .001$).

The data from the second trial demonstrate that one has considerable flexibility in the grade and source of reagents used in preparing the milk gelation test solutions. Undoubtedly, many other readily available brands of lye and detergents could perform equally well, although they were not included in this experiment. In any event, the test solution for the LMT was prepared at a

TABLE 2

Average CMT and LMT gelation test scores and within sample standard deviations and coefficients of variation

Test	Average gelation scores	Within sample	
		Standard deviation	Coefficients of variation
California Mastitis Test (CMT)	1.72	0.22	13%
Lye Mastitis Test (LMT)	1.77	0.36	20%

small fraction of the cost of the CMT and MQT test solutions. Furthermore, the procedure for preparing the LMT solution is simple, the ingredients are readily available, and the equipment used in preparing the test solution is present in most households.

EXPERIMENT II. THE EFFECTS OF DEOXYRIBONUCLEASE ON THE MILK GELATION TESTS FOR MASTITIS

The gelation of the CMT, MQT, and MMT were reminiscent of the behavior of deoxyribonucleic acid (DNA) in solution. The present experiment was designed to test the hypothesis that DNA of milk somatic cells was the material responsible for the gels.

Materials and Methods

A deoxyribonuclease (DNAse)[1] solution was prepared by dissolving 2 mg of DNAse in 2 ml of 0.008 molar acetate buffer and 0.025 molar $MgCl_2$ (pH 4.6).

The gels to be tested were obtained by adding the CMT, MQT, or MMT reagents to a known mastitic milk sample and then centrifuging for 15 minutes at 26,000 gravities. After centrifugation, the gels were removed and each divided into approximately two equal portions and placed in individual glass petri dishes. The pH of the gels was 11.5 and was adjusted to 5.3 by the addition of 6 N HCl. One-hundredth ml of the DNAse solution was then added to one portion of each sample and 0.01 ml of distilled water to the other portion (control). According to the Worthington Biochemical Corp., from whom the enzyme was

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[1] Worthington DNAse II.

purchased, the DNAse shows optimum activity at this lower pH. The pH was then adjusted to the original pH of 11.5 by the addition of one drop of 6 N NaOH. The trial was repeated for six different mastitic milk samples.

Results and Discussion

No gelation recurred in the DNAse treated sample, but a heavy gel recurred in the control sample after elevation of the pH to 11.5. The same results were observed for each of the six different mastitic milk samples.

The addition of trypsin[1], pepsin[2], proteinase[3], papain[4], and DNAse[5] had no effect on the viscosity of the gels as measured by viscometers. These findings and the enzymatic specificity of DNAse for DNA indicated that DNA of milk somatic cells was responsible for the gel in the gelation tests.

Since this work has been completed similar findings have been reported by Caroll and Schalm (10) for the CMT.

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[1] Worthington Biochemical Corp.

[2] General Biochemicals Corp.

[3] Nutritional Biochemicals Corp.

[4] Fisher Scientific Co.

[5] Worthington Biochemical Corp.

EXPERIMENT III. VARIATION OF ESTIMATED NUMBERS OF MILK SOMATIC CELLS STAINED WITH WRIGHT'S STAIN OR PYRONIN Y - METHYL GREEN STAIN

Review of Literature

Several researchers Guallini & Leali (16), Guallini & Vallis (17), and Varrier-Jones (46) have attempted to make differential counts of the leucocytes in milk as an indication of mastitis. Macadam (27) concluded that the proportion of granulocytes usually exceeded 70% of the total cells from acutely infected quarters, whereas it was usually less than 40% during mammary involution. Blackburn et al. (5) indicated that milk samples with a low total cell count generally contain less than 45% granulocytes and claimed that the differential cell count is a valuable criterion to confirm the conclusions drawn from the results of a total cell count, especially where doubts arise owing to the presence of bacteria in the milk.

Because of the opacity of milk, it is necessary to stain the leucocytes before microscopic observation. In the course of a study of milk leucocytes in the authors' laboratory, the commonly used stains such as Wright's (48), Newman's (33), Newman Modification (26), Broadhurst-Paley (9) and its modification (11) were used and found to be unsatisfactory because background staining masked the leucocytes, or because of a lack of nuclear-cytoplasmic

resolution within the leucocytes.

The results of the previous experiment showed that the CMT, MQT, and MMT gelation reactions were due to the DNA in the milk somatic cells. This fact indicated that a staining procedure specific for nucleic acid would be preferable to the previously used stains because the background would not be expected to take appreciable quantities of stain. Brachet (6, 7, 8) has shown that the stain methyl green is specific for DNA and that pyronin Y is specific for ribonucleic acid (RNA). Kaufmann (23), using a staining mixture composed of methyl green and pyronin Y, found that chromosomes stain blue-lavender, whereas nucleoli and RNA-containing cytoplasmic particles stain red.

Preliminary investigations in the author's laboratory indicated that the pyronin Y - methyl green stain resulted in considerably improved microscopic resolution of milk somatic cells. The backgrounds of smears stained with pyronin Y - methyl green were clear with a light lavender mottling. The cytoplasm of epithelial cells contained large quantities of pyronin Y-positive material, whereas agranulocytes contained small quantities and granulocytes contained none.

The purpose of this experiment was to compare the variation of estimated numbers of somatic cells in milk stained with pyronin Y - methyl green with that of cells stained with Wright's stain.

Materials and Methods

A milk sample was obtained from each of 27 Holstein cows in various stages of lactation. Fourteen of these cows were considered to have mastitis, based upon CMT observations. Within 30 minutes after obtaining the samples, four smears were prepared from each milk sample by the direct-smear method of Prescott and Breed (37), which consisted of transferring 0.01 ml of milk in a standard platinum loop to a microscope slide and distributing the milk over one square centimeter. The smears were stained with Wright's stain according to the procedure outlined by Schalm (41) (Appendix, Procedure I). The remaining two smears were stained with pyronin Y - methyl green stain according to the following procedures:

a) Fix in Carnoy's for ten minutes.

b) Hydrate for two minutes each in 50% ethyl alcohol, 30% ethyl alcohol, and distilled water.

c) Stain for six minutes in a freshly prepared solution of 0.50% pyronin Y (National Analine) and 0.30% methyl green (National Analine) in distilled water.

d) Immerse for three minutes in each of two changes of N-butyl alcohol.

e) Clear for three minutes in each of two changes of xylol.

f) Mount in 50% piccolyte dissolved in xylol.

After preparation, the smears were microscopically examined, using immersion oil, by a person who was unaware of the identity of the smear. The diameter of a microscope field was 0.178 mm, resulting in about 4,000 fields per smear (Appendix, Procedure II). The number of somatic cells per ml of each milk sample was estimated from the number in 25 fields randomly chosen from a smear and multiplied by 16,000. This constituted a count. The number of agranulocytes, granulocytes, leucocytes (the sum of agranulocytes and granulocytes), epithelial cells, and total somatic cells (the sum of leucocytes and epithelial cells) was determined in duplicate for each smear.

Five identical analyses of variance, one for each of the cell classifications, considered the following sources of variation: milk samples, stains, smears, counts, and an interaction between samples and stains. Samples were considered to be random and factorial, and stains to be fixed and factorial. Smears were considered to be random and nested within samples and stains, and counts to be random and nested within smears.

To compare the variation of determinations of cell numbers within each of the two staining procedures, the variance of duplicate smears and of duplicate counts were estimated for each stain for the number of total cells and the number of granulocytes. These variances were used to estimate the standard errors of determinations of

mean cell numbers for future milk samples with varying numbers of smears per sample and counts per smear, as shown in the following formula:

$$s_{\bar{x}} = \sqrt{\frac{s^2_{b:m}}{n_b} + \frac{s^2_{c:bm}}{(n_b)(n_c)}}$$

where $s^2_{b:m}$ is the smear variance, $s^2_{c:bm}$ is the count variance, n_b is the number of smears per sample, and n_c is the number of counts per smear. The standard errors were then used to predict the 95% confidence intervals for determinations of mean cell numbers as follows:

$$95\% \text{ confidence interval} = (2)\ (t_{.05})\ (s_x),$$

where $t_{.05}$ has $n_b n_c - 1$ degrees of freedom. Similar calculations were not made for the other three cell classifications because they have not been extensively used in mastitis research.

Results and Discussion

The average number of cells for each of the five cell classifications as determined after staining with Wright's stain or with pyronin Y - methyl green are listed in Table 3 with their corresponding coefficients of variation. The coefficients of variation were determined by taking the square root of the sum of the estimated smear and count variances and dividing by the mean. The milk sample variance was excluded from these estimates because the samples included in the present experiment

would not necessarily be representative of those chosen for a future experiment where, for example, one may begin with either infected cows or cows with no history of mastitis. Consequently, the coefficients of variation in Table 3 are minimal estimates. A summary of the probabilities (p_I's) obtained in the analysis of variance for each of the five cell classifications is presented in Table 4.

TABLE 3

Averages and coefficients of variation for each milk cell classification with Wright's stain or pyronin Y - methyl green

Cell classification	Wright's stain		Pyronin Y - methyl green	
	$\bar{x}$	C.V.	$\bar{x}$	C.V.
	(x 10^4)	(%)	(x 10^4)	(%)
Agranulocytes	8	62	15	100
Granulocytes	306	58	344	41
Leucocytes	314	57	359	41
Epithelial cells	64	33	63	30
Total cells	378	49	422	36

The number of cells determined in smears stained with pyronin Y - methyl green was considerably larger than that determined in smears stained with Wright's stain for agranulocytes ($P \cong .13$), granulocytes ($P \cong .07$), leucocytes ($P \cong .03$), and total cells ($P \cong .03$), but not for epithelial cells ($P \cong .50$). Apparently, the epithelial cells'

are equally recognizable in the two stains, a conclusion which is supported by the similarity of the coefficients of variation of epithelial cell counts. In contrast, these data suggest that a considerable portion of the leucocytes was masked by the background of those smears prepared with Wright's stain. Since the interaction between samples and stains did not approach significance for granulocytes, leucocytes, or total cells, we may conclude that where these cells are of primary importance one may expect to obtain larger cell numbers in most milk samples when the cells are stained with pyronin Y - methyl green.

TABLE 4

Level of significance (P_I) for each measurable source of variation for each milk cell classification analysis.

Source of variation	Degrees of freedom	Cell classification				
		Agranulocytes	Granulocytes	Leucocytes	Epithelial cells	Total cells
Milk sample	26	.01	.01	.01	.01	.01
Stain	1	$\cong$ .13	$\cong$ .07	$\cong$.03	.50	$\cong$ .03
Interaction	26	.01	.50	.50	$\cong$ .16	.50
Breed smears	54	.01	.01	.01	.01	.01

$\cong$ Approximately equal to

As expected, milk samples differed significantly for each of the five cell classifications. However, duplicate smears also differed significantly ($P < .01$) in

each analysis, a result that was not anticipated in view of the opinions of Blackburn et al. (5), McKenzie (31), Meigs et al. (32), and Waite & Blackburn (47) that the "Breed" smear method gave very consistent results.

Estimated smear and count variances for both stains are shown in Table 5 for granulocytes and for total cells. Although, asshown above, significantly more cells were counted in the pyronin Y - methyl green stain, this stain resulted in an approximately 25% reduction in the variance between duplicate smears and in an approximate 50% reduction in the variance between duplicate counts when compared with Wright's stain. Also, the fact that the smear variance is at least two to four times larger than the count variance suggests that some major errors are inherent in the smear technique used. For instance, the use of a platinum wire loop to deliver 0.01 ml of milk could be a major source of variation contributing to the large smear variance.

TABLE 5

Estimated smear ($s^2_{b:m}$) and count ($s^2_{c:bm}$) variances for granulocytes and total cells in milk stained with Wright's stain or pyronin Y - Methyl Green

Variance Component	Wright's stain		Pyronin Y - Methyl Green	
	Granulocytes	Total cells	Granulocytes	Total cells
	(x 10^8)			
$s^2_{b:m}$	22,702	25,191	16,152	19,580
$s^2_{c:bm}$	9,241	9,883	4,003	3,975

Expected 95% confidence intervals for mean numbers of milk somatic cells which are obtained from varying numbers of smears and counts are tabulated in Table 6. If one count on the total cells is made on each of two smears from a milk sample, the expected 95% confidence interval is $3,354 \times 10^4$. In other words, in about 95 out of 100 such observations, μ will be within the range $\bar{x} \pm 1,677 \times 10^4$. This range is far too large, because most researchers agree that milk with 50×10^4 to 100×10^4 cells per ml is mastitis positive (22, 36).

Although inspection of Table 6 reveals somewhat smaller values for the pyronin Y - methyl green stain, the differences between the stains are small relative to the magnitude of the confidence intervals. Furthermore, these data show that the number of smears has more influence on the magnitudes of the confidence intervals than does the number of counts. If one were to make an infinate number of counts of total cells on only one smear with Wright's stain, the 95% confidence interval would be about 623×10^4, indicating that elimination of the count variance does not bring the interval close to the important value of 50×10^4 cells. Consequently, it may be generalized that one should make upwards of 200 smears from a milk sample in order to obtain a 95% confidence interval of about 50×10^4.

In view of this evidence, it appears that the magnitude of the error variance must be reduced before

TABLE 6

Expected 95% confidence intervals for a mean number of milk cells obtained from differing numbers of smears per milk sample and counts per smear

Numbers of counts per smear	Number of smears per milk sample	Wright's stain		Pyronin Y - Methyl Green	
		Granulocytes	Total cells	Granulocytes	Total cells
		(x 10^4)			
1	2	3,202	3,354	2,542	2,770
	5	444	466	356	384
	20	168	176	134	142
	200	52	52	40	44
10	2	456	478	380	418
	5	278	290	230	254
	20	134	142	114	126
	200	44	44	36	40

estimations of milk cell numbers can be considered to be a practical indicator of the inflammatory state of the udder. One possible method of reducing the magnitude of the error variance would be to deliver the 0.01 ml volume of milk with a ten-lambda pipette, which would be expected to result in a reduced smear variance.

EXPERIMENT IV. FEULGEN-DNA IN MILK AS A MEASURE OF UDDER IRRITATION

Review of Literature

Babel (2) and Plastridge (36) state that leucocyte counts are generally regarded as being superior to other laboratory tests for diagnosing inflammation of the mammary gland. However, the previous experiment demonstrated that present methods for direct microscopic estimations of leucocyte numbers have serious limitations. Also, in view of the evidence presented in experiment II showing that DNA was the material responsible for the CMT reaction, it seemed likely that one of the standard DNA tests would also be useful for the detection of udder irritation.

The Feulgen nuclear reaction is commonly used as a histochemical test for DNA. The reaction was introduced by Feulgen and Rossenbeck (14) as a specific test for DNA and is dependent on the release of aldehyde groups from the deoxypentose sugar of DNA by acid hydrolysis with 1 N HCl at 60°C. The exposed aldehyde groups then react with the Schiff's reagent to produce a color reaction at the site of any DNA present. Such a reaction could be easily standardized and would therefore be more objective than the CMT.

The purpose of this experiment was to adapt the Feulgen-DNA test to milk and to determine its relationship to the concentration of somatic cells in milk.

Materials and Methods

Seventy-five milk samples were obtained from Holstein cows in various stages of lactation. Duplicate Feulgen-DNA and duplicate MQT determinations were performed as an indication of the milk somatic cell concentration within 30 minutes after obtaining the milk sample. The MQT was used as previously described. Milk samples were selected at random until 15 were obtained for each MQT score. Feulgen-DNA determinations were performed according to the following procedure:

a) Each milk sample (2 ml) was hydrolyzed with an equal amount of 1 N HCl at 60° for 24 minutes.

b) Schiff's reagent was then added in an amount equal to twice the volume of milk. Schiff's reagent was prepared by dissolving 1.00 g of basic Fuchsin[1] in 200 ml boiling distilled water, cooling to 50° C. adding 20 ml of 1 N HCl, colling to 24° C, and dissolving 2.00 g of sodium metabisulfite. This solution was stored in the dark for 10-15 hrs and then 0.5 g activated charcoal was added, the mixture stirred for one minute, and rapidly filtered

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[1] National Aniline C.I. No. 42500.

through Whatman No. 1 paper. The filtrate should be stored in a full, well-stoppered, dark bottle at 5° C. The solution should be colorless; development of a pink color indicates that it must be discarded.

c) Thirty minutes was allowed for color development.

d) The degree of inflammation was scored by comparing the color intensity of the treated milk sample with a standard color chart similar to the one shown in Fig. 1.[1]

Two smears were prepared from each of the 75 milk samples by the direct smear method of Prescott and Breed (37). The previous experiment on the direct microscopic estimation of number of somatic cells in milk, using a standard platinum loop in conjunction with the "Breed" smear, showed that duplicate smears were highly variable. In an attempt to reduce this error in the present experiment, a 10-lambda pipette was used in place of the platinum loop to transfer the milk to the microscope slides. Between samples the pipette was washed in detergent, rinsed with water, acetone, then dried with forced air. The smears were air-dried on a level surface for 24 hours and then stained with pyronin Y - methyl green by the procedure described in experiment III. The concentration

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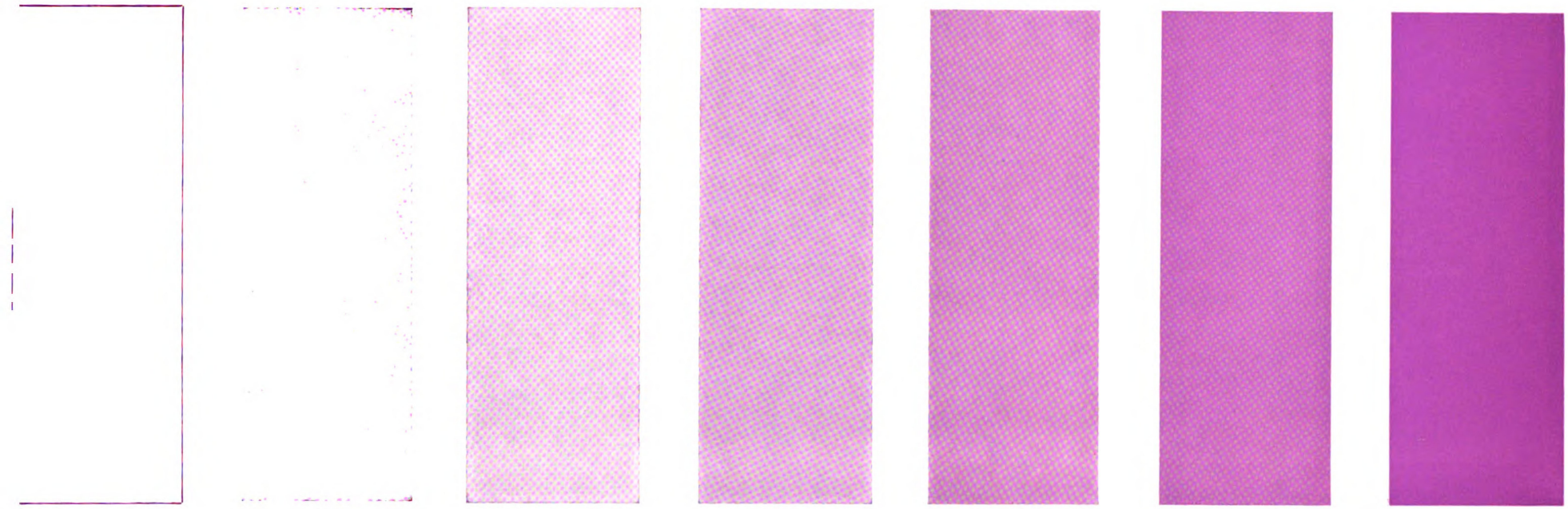
[1] A standard color chart is available upon request.

COLOR STANDARD FOR FEULGEN-DNA IN MILK

Department of Dairy
MICHIGAN STATE UNIVERSITY
East Lansing, Michigan



0 1 2 3 4 5 6

Directions for Use:

(1) Cut one half-inch hole in each color block.
(2) Mount chart in clear plastic folder.
(3) To score: Move sample behind color blocks to match color.

Figure 1. Standard color chart for Feulgen-DNA test

of somatic cells per milliliter of each milk sample was estimated from the number in 25 microscopic fields. The number of agranulocytes, granulocytes, leucocytes (the sum of agranulocytes and granulocytes), epithelial cells, and total somatic cells (the sum of leucocytes and epithelial cells) were determined on two smears from each milk sample by each of two persons who were unaware of the identity of the smears.

Results and Discussion

The correlation of Feulgen-DNA score with MQT score was 0.907 ($P < .01$). The standard deviation of duplicate Feulgen-DNA scores was 0.114, considerably less than the 0.230 for duplicate MQT scores. The coefficients of variation were 7 and 12% for Feulgen-DNA and MQT scores, respectively.

The average numbers of cells for each of the five cell classifications are listed in Table 7 with their corresponding coefficients of variation. The coefficients of variation were determined by taking the square root of the sum of the smear and count variances and dividing by the mean, for the reasons outlined in experiment III. These values were generally lower than those in Table 3 from slides prepared by means of a platinum loop, but were much higher than the value of 0.114 for duplicate Feulgen-DNA scores.

Each of the estimated variances for duplicate smears ($s^2_{b:m}$) was negative, and since negative variances are not possible these values are considered to be estimates of parameters which are very close to or equal to zero. These results were in direct contrast to the results of experiment III in which the smear variance accounted for at least two-thirds of the total within sample error. The reduced smear variation in the present experiment was probably due to the use of a 10-lambda pipette to transfer milk to microscope slides. The estimated variances for duplicate counts ($s^2_{c:bm}$) in the present experiment were two to three times larger than those in experiment III, probably because one person made

TABLE 7

Averages, coefficients of variation, and estimated smear ($s^2_{b:m}$) and count ($s^2_{c:bm}$) variances for each milk cell classification

Cell classification	$\bar{x}$	CV	$s^2_{b:m}$	$s^2_{c:bm}$
	($\times 10^4$)	(%)	($\times 10^8$)	
Agranulocytes	24	83	-266	666
Granulocytes	254	34	-544	7,882
Leucocytes	278	33	-1,866	10,434
Epithelial cells	72	40	-79	942
Total cells	350	32	-1,667	13,829

the duplicate counts in the earlier experiment, whereas in the present experiment, a count was made on each smear by each of two persons. In other words, count variance included person to person variance in the present experiment.

The correlations of the estimated number of each cell type with Feulgen-DNA score and with MQT score are tabulated in Table 8. Each of these correlations was significant ($P < .01$) and those for the relationship of Feulgen-DNA with cell numbers were consistently higher than those for MQT and cell numbers. However, the highest correlation of 0.876 between Feulgen score and total cells accounts for only 77% of the total variation, indicating that these relationships are low for prediction purposes.

TABLE 8

Correlation coefficients between somatic cell numbers and Feulgen-DNA or MQT scores

Cell classification	Feulgen-DNA score	MQT score
Agranulocytes	.805	.769
Granulocytes	.814	.730
Leucocytes	.834	.751
Epithelial cells	.816	.790
Total cells	.876	.799

Feulgen-DNA and MQT scores were plotted against the estimated number of total somatic cells per milliliter of milk, resulting in the curves shown in Figure 2. Since these relationships were not linear, an attempt was made to create a linear relationship by logarithmic transformation of Feulgen-DNA scores and total somatic cell numbers. It was necessary to code the Feulgen scores by adding the number one to each score to eliminate the score of zero. The curve resulting from a double logarithmic transformation of the coded data is illustrated in Figure 3.

The relationship between MQT scores and cell numbers was not transformed because of the subjectivity of the MQT scores. Although MQT scores are quite repeatable within a single person at any given time, they are much more variable between people because there is no standard for each score. This lack of objectivity limits the value of MQT in the prediction of cell numbers.

The correlation between logarithms of coded Feulgen-DNA scores and logarithms of estimated numbers of total somatic cells was 0.909 ($P < .01$), a value which, although higher than that for the non-transformed data, accounts for only 83% of the total variation. Nevertheless, the regression equation shown in Figure 3 should be useful for estimating total somatic cell numbers from Feulgen-DNA score. In view of the high variation (C.V.= 32%) associated with microscopic estimations of total

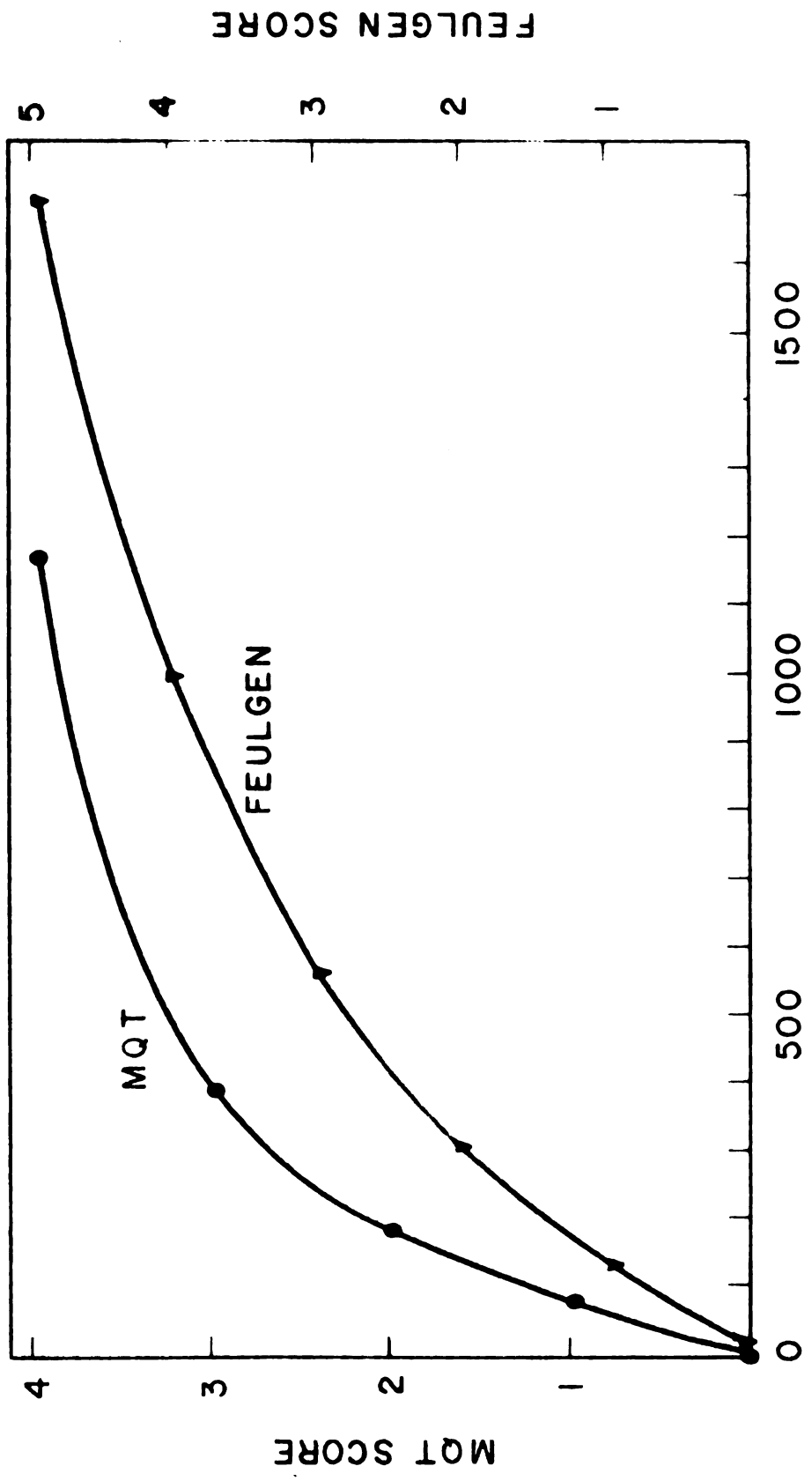


Figure 2. Relationship of concentration of total somatic cells to Feulgen-DNA score and to MQT score

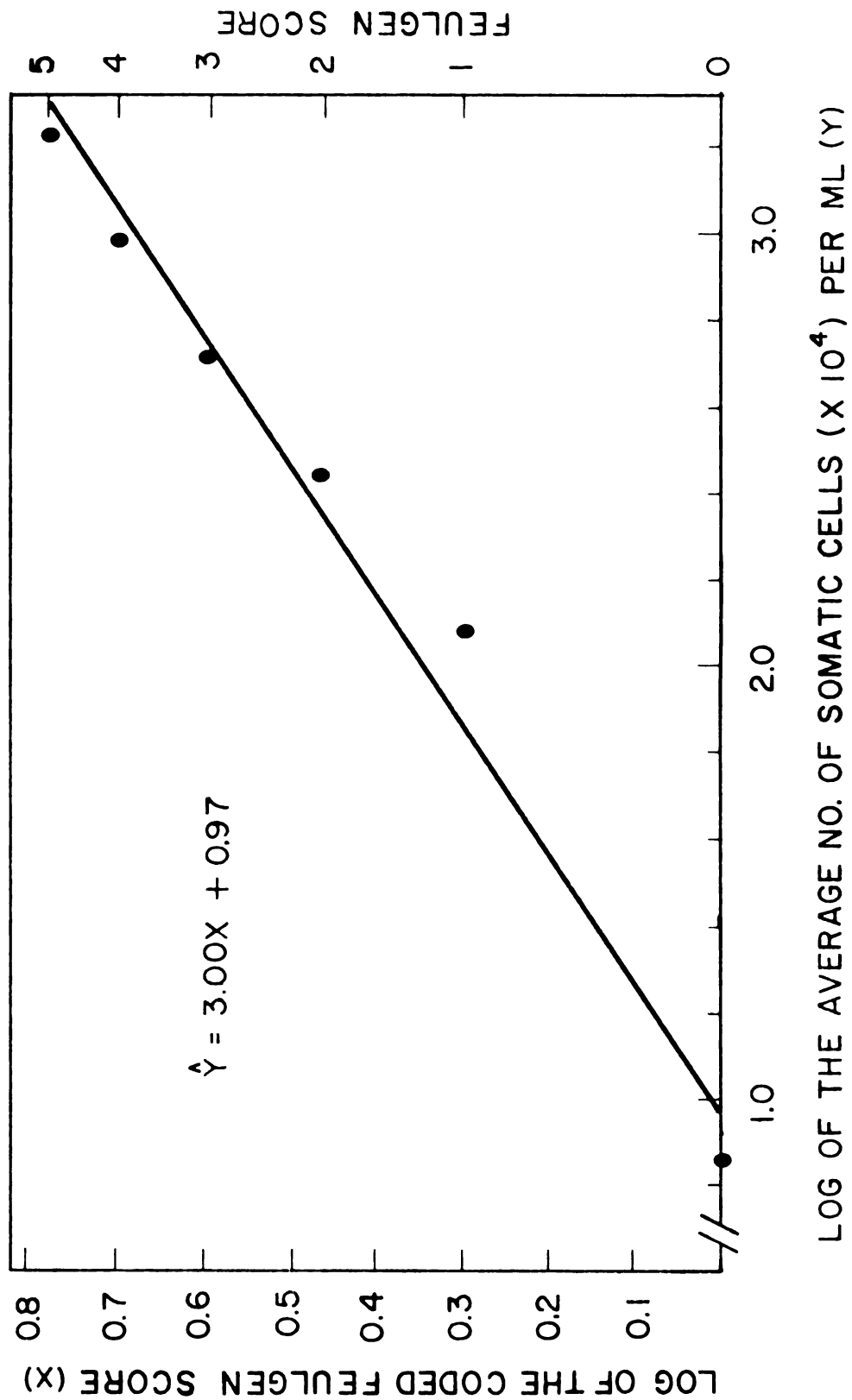


Figure 3. Regression of logarithm of average number of somatic cells (Y) on the logarithm of the coded Feulgen-DNA score (X)

cell numbers, this regression equation may be at least as accurate in estimating milk cell numbers as microscopic estimations in the manner routinely performed in most laboratories.

The colors in Figure 1 were selected from a large number of possibilities which ranged from white to the most dense color shown in Figure 1. The selection of the colors was arbitrary since it was based upon the ability to distinguish small differences in the intensity of this color. A preferable procedure, which would eliminate all subjectivity, would be to measure the intensity of the color of the Feulgen-treated milk samples by reflectance spectrophotometry. Although such a procedure would be expected to be more reliable, it would be more costly than the Feulgen scoring procedure used to obtain the Feulgen-DNA data in this experiment.

SUMMARY

An open formula gelation test for mastitis, the Michigan Mastitis Test (MMT), was developed which produced results equivalent to the CMT and MQT. In addition, the MMT ingredients can be purchased, and the reagent prepared with the aid of a pan balance. The average test scores for 91 samples of milk tested in duplicate with CMT, MQT, and MMT were 1.68, 1.76, and 1.81, respectively. The comparable within sample standard deviations were 0.33, 0.33, and 0.32, respectively. These data indicated that the results of the three tests are essentially equivalent and that one may choose among them on a basis of cost, availability, or personal preference.

In an effort to make the MMT open formula test solution more practical to prepare, a test solution was prepared using ingredients that are readily available. This test solution may be prepared with the equipment available in most households. Although the results of the use of this test were somewhat more variable than those for CMT, they demonstrate that one has considerable flexibility regarding the source and quality or purity of the ingredients of the test solutions employed in the mastitis gelation tests.

Since the gelation of the CMT, MQT, and MMT was reminiscent of the behavior of DNA, DNAse was added to

the gels with the result that the gels disappeared, indicating DNA was responsible for the gels.

In the course of a study of milk leucocytes, the commonly used stains were found to be unsatisfactory because background staining masked the leucocytes, or because of a lack of nuclear-cytoplasmic resolution within the leucocytes. In view of the evidence that DNA was the material responsible for the gelation in the CMT, MQT, and MMT, and since leucocytes are the primary source of nucleic acids in milk, the nucleic acid-specific stain pyronin Y - methyl green resulted in considerably improved microscopic resolution of milk somatic cells.

To compare the variation of estimated numbers of somatic cells in milk, four "Breed" smears were made from each of 27 milk samples, including 14 mastitic samples. Two smears were stained with Wright's stain and two with pyronin Y - methyl green stain. The average ($\times 10^4$) obtained from duplicate determinations of agranulocytes, granulocytes, leucocytes, epithelial cells, and total somatic cells were 8,306, 314, 64, and 378, respectively, for Wright's stain; and 15, 344, 359, 63, and 422, respectively, for pyronin Y - methyl green stain. Although the epithelial cells were equally recognizable in the two stains, a considerable portion of the leucocytes were masked by the background of the smears prepared with Wright's stain.

The pyronin Y - methyl green stain resulted in an approximate 25% reduction in smear variance and in an approximate 50% reduction in count variance when compared with Wright's stain. Expected 95% confidence intervals for determinations of mean cell numbers were computed from the estimated variance components with varying numbers of smears and counts. These values generally indicated that one should make upwards of 200 smears from a milk sample to obtain a 95% confidence interval of approximately 50 x 10^4 cells per ml of milk, a result leading to the conclusion that the magnitude of the error variance must be reduced before estimations of milk cell numbers can be considered to be a practical indicator of the inflammatory state of the udder.

In view of the evidence that DNA was the material responsible for the gelations in the CMT, MQT, and MMT reactions the Feulgen-DNA test was modified as follows: A milk sample was hydrolyzed with an equal amount of 1 N HCl at 60° C for 24 minutes. Schiff's reagent was then added in an amount equal to twice the volume of milk. Maximum color development occurred within 30 minutes after the addition of Schiff's reagent. The developed color was compared with a color chart which contained seven colors, corresponding to seven possible scores for udder inflammation.

To determine the reliability of the Feulgen-DNA

test in estimation of the concentration of milk somatic cells, duplicate MQT's were made for each of 75 milk samples. Milk somatic cell concentrations were determined in duplicate from "Breed" smears after transferring milk to each of two microscope slides with a 10-lambda pipette and staining the smears with pyronin Y - methyl green stain.

The correlation of the number of each cell type with Feulgen-DNA score was consistently higher than with MQT score, but the highest correlation of 0.876 between Feulgen-DNA score and total somatic cell numbers accounted for only 77% of the total variation. Plotting the observed values revealed a curvilinear relationship, which became linear when both variables were transformed to logarithms. The correlation of the logarithm of the coded Feulgen-DNA scores with numbers of total somatic cells was 0.909, including that Feulgen-DNA has practical value in the estimation of somatic cell numbers in milk.

The coefficient of variation for Feulgen-DNA scores was 7%, much less than the 32% for the microscopic estimations of somatic cell concentration, indicating that the Feulgen-DNA socres may be at least as accurate in estimating milk cell numbers as microscopic estimations.

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APPENDIX

Procedure I: Staining with Wright's stain.

a) Defat and mix the milk smear in acetone-free methyl alcohol for 2-3 minutes.

b) Place in Wright's stain for 3-5 minutes.

c) Slides are removed from the stain directly into phosphate buffer to which has been added 1 part of Wright's stain solute to 10 parts of buffer. After 3-4 minutes in the buffer the slides are briefly rinsed in clear water and allowed to dry without blotting, at room temperature. The phosphate buffer is prepared by diluting 3.8 g Na_2HPO_4, and 5.4 g of KH_2PO_4 to 1,000 ml of distilled water.

Procedure II: Method Used in Finding the Area of a Microscopic Field.

By using a stage micrometer the diameter of a field was found to be 17.8 mm (0.0178 cm). Area equals $3.1416 \times (0.0089)^2 = 0.00025$ sq. cm or 1/4000 sq. cm.

The milk volume represented by each field equals $1/4000 \times 1/100 = 1/400{,}000$ ml. Each cell seen in a field taken at random equals 400,000 cells/ml of milk. In examining 25 fields, each cell would then represent 16,000 cells.