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ABSORPTION-SPECTROPHOTOMETRIC DETERMINATION OF DNA (DEOXYRIBONUCLEIC ACID) IN MILK AD MODUM FEULGEN*)

ACCURACY OF ANALYSIS, SPECIFICITY, CORRELATION TO CONTENT OF CELLS

By
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One of the symptoms of inflammation of an organ is emigration into the tissue of leucocytes, another reaction is increase of the capillary permeability in the affected area resulting in exudation of blood plasma in the tissue (*Menkin* 1956). This situation is the same for aseptic as well as for septic inflammation.

Exudation and emigration in the udder have the effect that leucocytes and exudate permeate into the alveoles and the milk ducts where they become mixed with the milk produced in the secretory cells.

The criterium for the presence of mastitis in a quarter therefore must be a demonstration of leucocytes or exudate in the milk.

Demonstration of leucocytes in milk can be made by microscopic count of the number of intact stainable cells, by electronic counting of all particles in the milk of the same size as leuco-

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cytes, or by quantitative determinations of DNA, characteristic of the cell nuclei.

Demonstration of exudate in the milk is made by examination of the content of substances present in blood in concentrations different from those in milk, e.g. chloride, lactose, potassium, sodium and others.

For demonstration of inflammation in the udder efforts have mainly concentrated on the determination of leucocytes in the milk. Up till recent years this determination was made exclusively by direct microscopic counts or by catalase test. In the last decennium the determination of DNA in the milk has gained wide propagation, partly according to the Whiteside- and the CMT methods using a variety of modifications, partly as chemical colour reactions: Feulgen reaction and Indolhydrochloric acid reaction.

During the last few years a number of studies have been made on the Feulgen reaction for the determination of the DNA-content in milk. The colour reaction has been estimated partly by comparison with a colour chart, partly measured spectrophotometrically.

By comparison with the colour chart high correlation coefficients were found between log cell number and log Feulgen reaction: 0.909 (*Paape et al.* 1964), 0.98 (*Hauke & Lüttich* 1966), and 0.85 (*Hauke* 1967).

By reflectance photometry a correlation coefficient of — 0.93 was found (*Paape et al.* 1965) between reflectance percentage and cell content.

Hauke found a coefficient of variation of 5.8 %, and *Paape et al.* (1965) found 13 % with the colour chart method. By reflectance photometry *Paape et al.* (1965) found a within-sample coefficient of variation of 1 %.

In spite of the strong correlation between the cell content in the milk and the Feulgen reaction *Whittlestone & de Langen* (1965) express their doubts that the Feulgen reaction is a DNA-reaction, *de Langen* (1967) also rejects Schiff's reagent as applicable for the determination of DNA in milk. The reason for this uncertainty regarding the reaction specificity is found in studies made by *Widstrom* (1928) who found that only DNA-quantities above 500 µg were demonstrable by Feulgen reaction. This quantity corresponds to a cell content in the milk of 70 million per ml.

Contrary to the above *Hauke* finds that the addition of 5 µg of DNA per ml to cell-free milk is sufficient to cause Feulgen reaction.

As an objective and accurate method of examination for the determination of the cell content in milk is desirable, the following examinations have been performed to obtain a broader basis of evaluation of the applicability of the Feulgen reaction.

METHODS AND OBSERVATIONS

The Feulgen reaction was performed as described by *Paape et al.* (1964) with the modification that 1 ml of milk was used:

1. Hydrolysis of milk sample (1 ml) using equal amounts of 1 N-HCl at 60°C for 24 min.
2. Addition of 2 ml of Schiff's reagent.
3. Storage for 1 hr. for development of colour reaction.

The blank used for absorption measurements was carried through the same procedure, only with the addition of 2 ml of water instead of Schiff's reagent.

Schiff's reagent was also prepared according to the procedure described by *Paape et al.* (1964):

1. Solution of 1 g basic fuchsin in 200 ml boiling, distilled water.
2. Cooling to 50°C.
3. Addition of 20 ml 1 N-HCl.
4. Cooling to 25°C.
5. Addition of 2.0 g sodium metabisulfite.
6. Storage of the mixture at room temperature for 10—15 hrs.
7. Addition of 0.5 g active charcoal.
8. Stirring for 1 min.
9. Quick filtration through Whatman No. 1 filter paper.
10. Storage in a dark bottle at 4°C.

Absorption spectrophotometric measurements were performed using a Beckmann spectrophotometer, model B.

The following standard procedures were used:

Insertion of blank. Adjustment of the deflection of the pointer at 565 nm to 0.1 at the absorption scale of the apparatus by altering the width of the light slit. Shift to measuring at 485 nm. Observation of the blank absorption at this wave length. Insertion of the reaction mixture. The adjustment of width of slit for the absorption of sample at 485 nm is the one observed for blank at the same wave length. Shift to 565 nm. The corrected result of measurements then appears from the deflection at the scale — 0.1. This result is multiplied by 100 in order to have a convenient scale of figures.

The initial adjustment of the absorption of the blank at 565 nm to 0.1 instead of to 0.0 is made to avoid damage of the apparatus due to the maximum deflection, when the wave length is changed from 565 to 485 nm. Thus it is made possible to carry out the entire measuring of the milk sample without closing the slit.

The relation between the absorption measurements used in the present study and the reflectance photometric measurements applied by *Paape et al.* (1965) are

$$\text{Absorption} = \log \frac{1}{\text{reflection}}$$

The use of absorption measurements has the advantage that the correction to be made at transition between the areas of sensitivity of the spectrophotometer is significantly simplified. In the reflectance photometric determinations the result of measurements from one sensitivity area must either be divided or multiplied by $\sqrt{10}$, before comparison can be made with the result from another area. In absorption measurements addition or subtraction by 0.5 is required dependent on up or down movement in the sensitivity area.

The absorption results found are multiplied by 100. This gives a convenient working scale as shown in Table 1, in which correlating values of absorption $\times$ 100 and reflectance percentage are listed.

Table 1. Correlation between absorption $\times$ 100 and reflectance percentage.

Absorption $\times$ 100	0	1	2	3	5	10	20	30	40
Reflectance %	100	98	96	93	89	80	63	50	40

The colour in the Feulgen reaction has maximum absorption at wave length 565 nm. Measurements of the absorption differences between blank and reaction mixture at 565 nm sometimes yielded negative results, when it was anticipated that the reaction was 0 or slightly positive. Likewise, false positive measuring results might occur, i.e. moderate positive measuring results, with absence of colour in the reaction mixture. These factors are assumedly due to minor differences in the pipetting or in the intensity of shaking between blank and reaction mixture. The intensity of shaking has a certain influence on the size of the

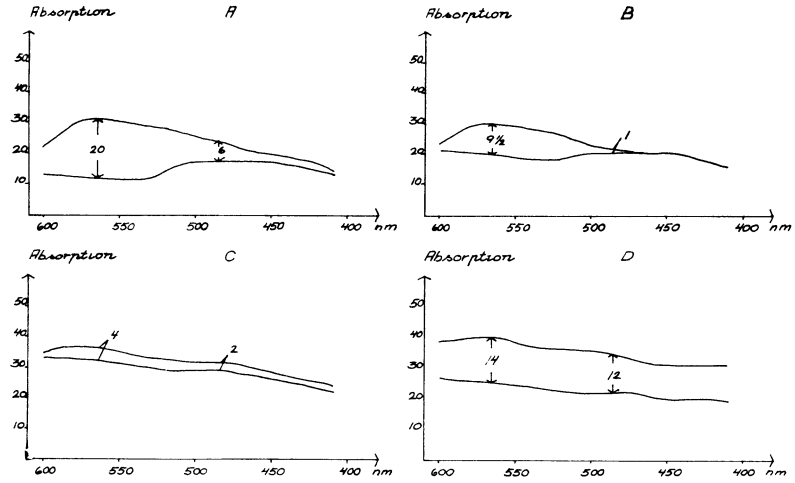


Figure 1. Absorption of reaction mixture and blank at various wave lengths using $MgCO_3$ as reference.
(The top curve in each Figure represents the reaction mixture, the lower curve the matching blank. The cell content in the milk sample is A: 2.6 millions, B: 1.2 millions and C and D below 100,000 per ml).

casein coagula in the suspension and on the admixture of air bubbles. Both of these factors influence the absorption.

These "false" results made a correction of these differences in "background absorption" desirable. A method was developed, with a special view to background absorption, by means of which measurements were made of the absorption for correlating blank and reaction mixture with a white reference ($MgCO_3$). In Fig. 1 the absorption is outlined for 4 milk samples at varying wave lengths.

It appears from the curves that the difference between absorption in blank and reaction mixture remains constant at approx. 490 nm. Because of instrumental advantages 485 nm was selected as the correcting wave length.

Fig. 1A shows measuring results for a milk sample of a cell content of 2.6 millions per ml. Registration of the absorption difference between blank and reaction mixture at 565 nm only yields a reaction of 20. Correction of the absorption differences at 485 nm gives a reaction of 14.

A milk sample of a cell content of 1.2 millions per ml is the basis of Fig. 1B. Before correction the reaction is 9½, after

correction 8½. The measuring results for 2 milk samples, both of a cell content of under 100,000 per ml, are shown in Fig. 1C and D. The reaction mixture and blank in the case of the milk sample illustrated in Fig. 1C were shaken as uniformly as possible. In the experiment, forming the basis of Fig. D, shaking of the reaction mixture was insufficient, whereas the tube containing the blank had been shaken sufficiently. It will be seen that insufficient shaking caused a very high increase in the absorption difference between reaction mixture and blank at 565 nm. This difference disappears with correction of the measuring result at 485 nm. This means that the Feulgen reaction after correction remains the same, whereas prior to correction it was 4 and 14 for C and D respectively.

In order to eliminate, if possible, the use of blanks the absorption differences between 565 and 485 nm were measured on the reaction mixture. The reference employed was $MgCO_3$. The measuring results were compared to the cell content, but due to the varying yellow-colouring of the milk samples (fat content) the correlation was poor. Absorption is higher for the yellow colour at 485 nm than at 565 nm, which means that the absorption differences are poor, when the milk is yellow-coloured.

Accuracy of analysis

On the basis of the above examinations the correction for "background absorption" at measurements at 485 nm proves applicable.

In order to compare the accuracy of analysis with or without correction both methods were employed in repeated examinations of the same milk samples. Using $MgCO_3$ as reference, determination was made of the absorption at 565 and 485 nm in 40 reaction mixtures and 20 blanks prepared on the same milk sample.

Each individual measuring of the absorption at 565 nm in the reaction mixture can be compared to each measuring of the absorption in blank at 565 nm (the non-corrected method).

Each individual measuring of the absorption difference between reaction mixture and blank at 565 nm can be compared to each measuring of the absorption difference between reaction mixture and blank at 485 nm (the corrected method).

Table 2. Comparative measurings using corrected and non-corrected method.

Sample no.	Non-corrected			Corrected		
	a	s	s %	a	s	s %
1	24.70	2.30	9	15.60	1.00	7
2	16.82	2.29	14	11.77	1.02	8
3	9.85	1.42	14	9.00	0.84	9
4	7.26	1.23	15	5.62	0.62	11
5	5.38	1.30	24	4.65	0.54	12
6	4.75	1.05	22	3.67	0.69	19
7	3.14	1.25	40	2.24	0.47	21
8	0.94	1.33	141	1.80	0.49	27

s = standard deviation a = average reaction

Table 2 shows the results of measurings on 8 different milk samples.

In comparing the standard deviations, expressed in percentage on the average reaction, these appear to be smaller with the corrected method.

The average measuring results are also found to be smaller with the corrected method.

With the purpose of studying further the correlation between the two methods measurements were performed on 131 milk samples of varying cell content (Fig. 2).

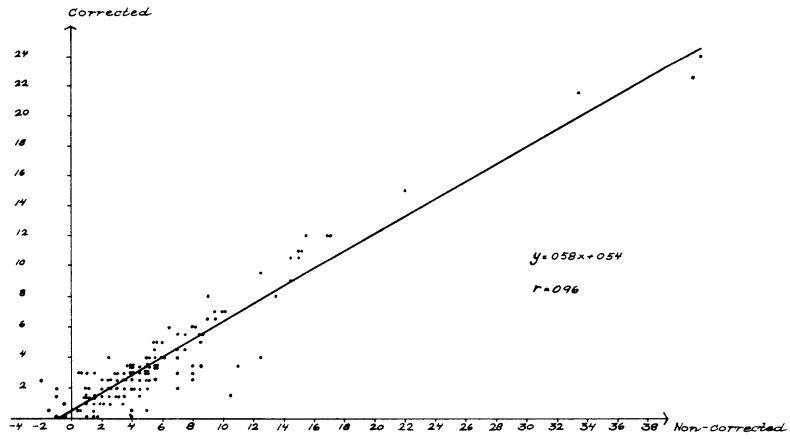


Figure 2. The relationship between corrected and non-corrected measuring results.

From the figure it appears that a linear relation exists between the results of the two methods according to the equation $y = 0.58x + 0.54$. The coefficient of correlation of 0.96 shows a very high correlation between methods, but it will be seen from the figure that the negative results are eliminated at correction and that the false positive results (e.g. 10½, 1), (11, 3½) and (12½, 4) also have been reduced significantly. It was observed that the high "false" results with the non-corrected method did not correlate with a high cell content in the milk. Neither did the colour intensity observed with the naked eye agree with the high result of measuring.

From Fig. 2 as well as from the coefficient of regression (0.58) can be deducted that the absorption differences found between reaction mixture and blank with the corrected method are smaller than those with the non-corrected method, but this minor differentiation of measuring results is sufficiently compensated for by elimination of the false results.

In the following the total number of measuring results refer to the corrected method only.

Comparison of accuracy of analysis by direct microscopic cell counts and Feulgen reaction

Cell counts and Feulgen reaction were carried out on the same milk samples. The procedure of the cell count determination is the one described by *Klastrup* (1960). The cell counts were made at varying working factors. Sixteen smears were made of the same milk sample, and the cell content in each of these smears was determined at working factors 55,000, 27,500, 18,330, 13,750 and 9,160.

The Feulgen reactions were measured by determination of the absorption against magnesium carbonate in 20 blanks and 40 reaction mixtures.

The curves in Fig. 3 represent the standard deviations of the cell counts in % of the average. It appears that the latter are highest at low cell numbers and high working factors. These results correspond to those found by *Schneider & Jasper* (1966). On the curve an "○" indicates the standard deviations for the Feulgen reaction. Judging from these results the standard deviations in the direct microscopic cell count and the Feulgen

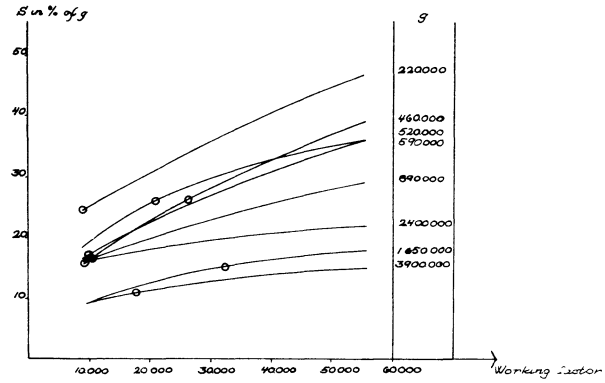


Figure 3. Comparison between standard deviations at Feulgen reaction and cell counts at various working factors. (s = standard deviation for cell counts, g = cell number determined at working factor 550. "○" on the curve indicates the standard deviation in % of the Feulgen reaction).

reaction are equal, when the cell count is performed at a working factor of 17,000.

The standard deviations for the Feulgen reaction indicated in Fig. 3 result from calculation on the basis of the figures in Table 2 by means of the equation of regression of Fig. 6.

Example: Sample no. 2 in Table 2 has a Feulgen reaction of 11.77 ± 1.02, which by calculation using the equation of regression of Fig. 6 yields 2.5 millions $\pm \begin{matrix} 400{,}000 \\ 300{,}000. \end{matrix}$ The average of 350,000 is 14 % of 2.5 millions.

This method of calculation appears to be the most reasonable in comparing the accuracy of analysis.

Addition of DNA to cell-free milk

With the purpose of studying the sensitivity of the Feulgen reaction to the content of DNA in milk, varying quantities of DNA (Sigma, type V) were added to a cell-free milk sample. The addition ranged from 3.4 µg to 200 µg per ml of milk. According to *Sandritter et al.* (1960) one diploid cell contains about 7×10^{-12} g DNA. This means that 1 µg of DNA corresponds

to about 145,000 cells. The concentration of the DNA added therefore corresponds to a theoretical cell content ranging from 500,000 to 29 millions per ml. In Fig. 4 the ordinate represents the measuring values of the Feulgen reaction on a logarithmic scale. The abscissa represents the µg of DNA added, also on a logarithmic scale.

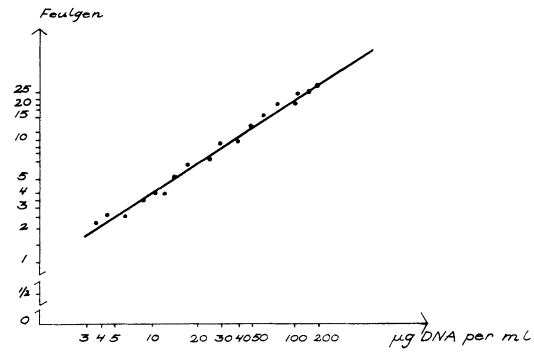


Figure 4. The relationship between log Feulgen reaction and log added DNA quantity.

The individual results are the average of the double determinations.

It appears that there is a linear relationship between log Feulgen reaction and log DNA-addition. The coefficient of correlation of 0.98 shows a very strong linear relationship between log Feulgen reaction and log added quantity of DNA in milk.

Examination of Schiff's reagent

In the examination of the Feulgen reaction on milk samples it was found that some of the preparations of Schiff's reagent gave less reactions than others. This was the case although exactly the same procedure was observed for the preparation. In order to examine these differences in intensity of reaction, each individual product of Schiff's reagent was examined against the above mentioned series of cell-free milk samples to which had been added varying quantities of DNA. The regression lines between log Feulgen reaction and log DNA content of varying quantities are drawn in Fig. 5.

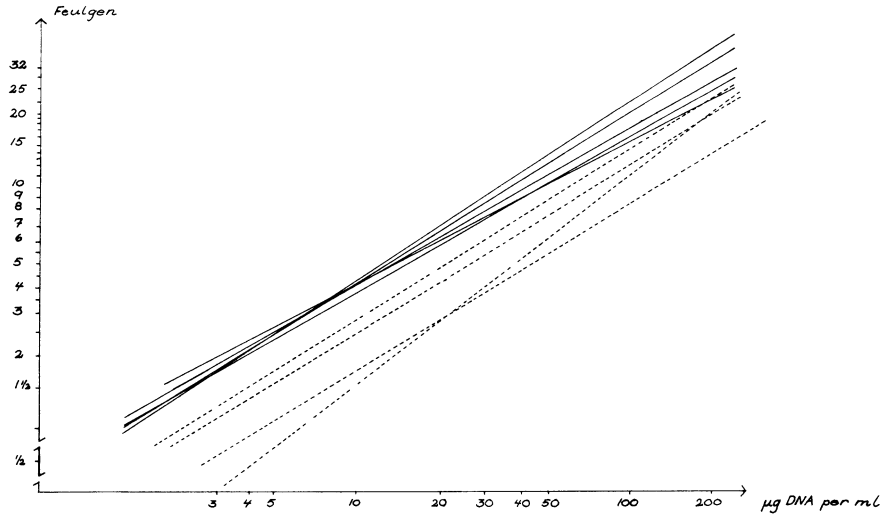


Figure 5. The relationship between log Feulgen reaction and log DNA quantity added at various preparations of Shiff's reagent.
(———— Basic fuchsin, -------- Diamond fuchsin).

In the preparation of Schiff's reagent two different dyes were employed: Basic fuchsin*) and diamond fuchsin**). It can be seen that results have been most constant with basic fuchsin, also, the reactions were highest with this dye. The individual measuring results are the average of double determinations. A calculation of the coefficients of correlation between log Feulgen reaction and log DNA content yielded figures between 0.97 and 0.99.

Because of the great differences in intensity of reaction and the weaker reactions with diamond fuchsin it was preferred to use basic fuchsin. Diamond fuchsin was only used for the examinations, on which Fig. 8 and partly Table 3 are based.

Correlation between Feulgen reaction and cell content

As illustrated in Fig. 3 and Table 2 there are inaccuracies in the determination of cell count as well as in the determination of DNA ad modum Feulgen. In order to study the correlation

*) Basic fuchsin, Allied Chemical No. 719. C I No. 42500.
**) Diamond fuchsin, Merck, S. Nr. 780. C I No. 42500.

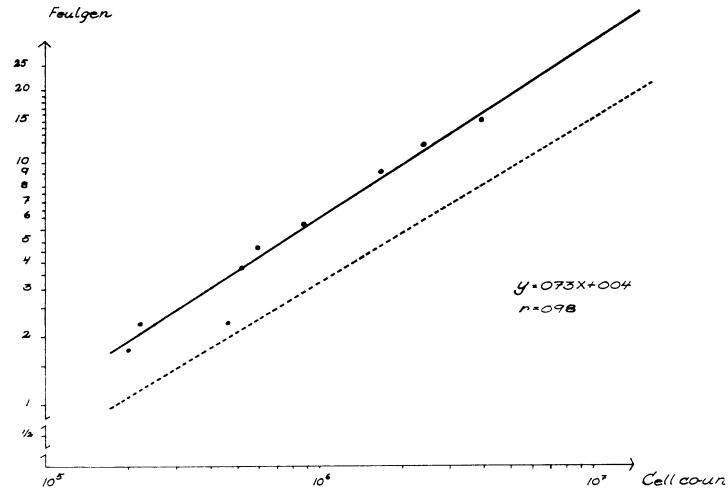


Figure 6. The relationship between log Feulgen reaction and log cell count. (y = log Feulgen reaction, x = log cell count/10^5. The Feulgen reaction is determined as the average of 800 measurements, the cell count determined at working factor 550. - - - - - - - - The regression between log Feulgen reaction and log DNA quantity added is calculated with 145,000 cells per g DNA).

between the two methods it is necessary to render these inaccuracies as small as possible, i. e. to make the determination of cell counts with small working factor and repeat the Feulgen reaction several times on the same milk sample. In the milk samples forming the basis of Fig. 6, the cell count was determined with a working factor of 550. The Feulgen reaction was determined 800 times on each sample (according to same principle as mentioned in the comments to Fig. 3).

The milk samples used in the test are prepared from a mixture of milk of various origin and of various cell content.

In Fig. 6, as in the following figures, the Feulgen reactions are plotted in a logarithmic scale on the ordinate and the cell counts in a logarithmic scale on the abscissa.

The figure shows a linear correlation between log Feulgen reaction and log cell count of the equation $y = 0.73x + 0.04$, where y is log Feulgen reaction and x is log cell count/10^5. The coefficient of correlation is 0.98.

In comparison to this accurate but time-consuming method, examinations were made of the same correlation, when the Feul-

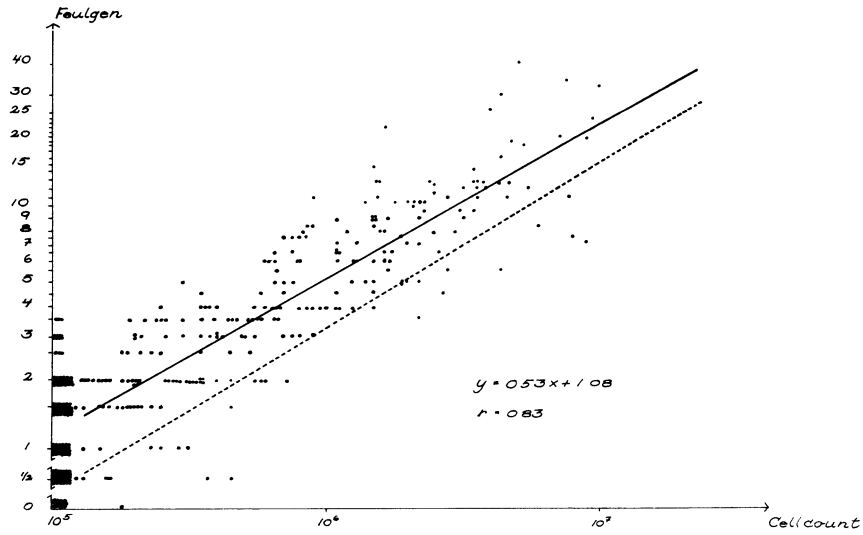


Figure 7. The relationship between log Feulgen reaction and log cell count. (y = log Feulgen reaction, x = log cell count/10^4. The Feulgen reaction is determined by one measuring on each milk sample, the cell count determined at working factor 20,000. -------- The regression between log Feulgen reaction and log DNA quantity added (calculated to cells)).

gen reaction was determined once on each milk sample, and the cell count was determined at a working factor of 20,000 (Fig. 7).

All cell counts below 100,000 per ml are marked as 100,000. In this method, with inaccuracies in both of the tests, the coefficient of correlation was reduced to 0.83. The equation of regression is $y = 0.53x + 1.08$, where y is log Feulgen and x is log cell count/10^4. It has been shown previously (Fig. 3) that the standard deviations are of the same order of magnitude in the determinations of Feulgen reaction and cell content at a working factor of 20,000. The inaccuracies in the determination of Feulgen reaction and the cell count determination therefore appear to have contributed equally to reduce the coefficient of correlation from 0.98 to 0.83, which means that the coefficient of correlation between the real cell content of the milk sample and the Feulgen reaction determined in a single measuring must be around 0.90.

Table 2 shows that the standard deviations, expressed in percentage, of the Feulgen reaction are higher the smaller the re-

action is. The 2nd decimal in the coefficient of correlation subsequently is affected by a possible prevalence, in the present test material, of milk samples of high or low Feulgen reaction and cell count.

The dotted lines in Figs. 6 and 7 represent the regression between log Feulgen reaction and log added DNA. (Lines from Figs. 4 and 5).

1 μg of DNA represent, as mentioned above, 145,000 cells.

Using this figure as calculating factor, the line of regression from Fig. 5 is transferred to the coordinate system in Figs. 6 and 7, employing the line of regression which corresponds to the relevant Schiff's reagent. It appears from Fig. 6 as well as from Fig. 7 that the results of measuring, obtained by examination of cell-containing milk, are higher than those theoretically anticipated.

Measurements of milk of various origin

In the examination of quarter milk samples (foremilk) the suspicion was aroused that a possible difference in the intensity of the Feulgen reaction might be dependent on whether the milk originated from a quarter with mastitis or from a quarter, in which the cell content in the milk was accounted for by the lactation stage or the age of the cow. In the present study the criterium for mastitis was: Simultaneous findings of udder-pathogenic bacteria and a cell content which was higher than that of the other quarters of the cow. Fig. 8 shows a differentiation of the measuring results according to mastitis status.

It appears that there is a strong tendency to higher Feulgen reactions in milk samples originating from quarters of mastitis than in milk from quarters of a "physiological" cell content, the number of cells being at the same level for the two categories.

Table 3 presents the Feulgen reactions of the foremilk samples of various origin. The Feulgen reaction is calculated as the average reaction within different cell count intervals.

The foremilk samples, forming the basis of Table 3, originate from 3 herds, in which, at an interval of 4 weeks, samples were taken from the total number of cows immediately before the evening milking. Therefore it was possible to distribute the reactions into the following 4 groups:

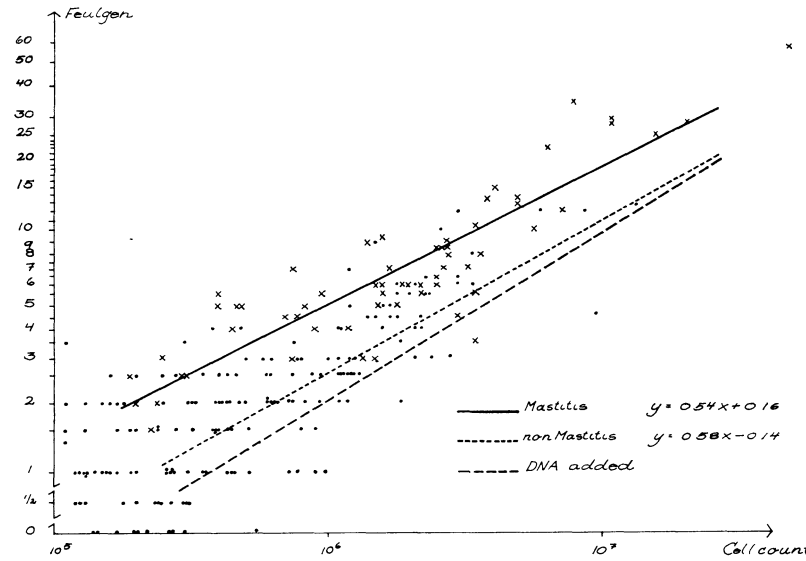


Figure 8. The relationship between log Feulgen reaction and log cell count, differentiated according to status of mastitis (y = log Feulgen reaction, x = log cell count/10^5. • = foremilk samples from quarters with physiological cell count, × = foremilk samples from quarters suffering from mastitis).

A. Reactions from quarters, in which the cell content because of stage of lactation and age must be considered physiological, or in which cell content was found in an examination 4 weeks earlier, although no udder-pathogenic bacteria could be demonstrated.

B. Reactions in quarters, which showed an increased cell count compared with the result of the examination 4 weeks previously, and in which the increased count could not be explained by the lactation stage.

C. Cell content in quarters, in which infection with mastitis-causing bacteria was determined, which infection also had been present at the examination 4 weeks previously.

D. Cell content in quarters, which were found infected with mastitis-causing bacteria, and which had not been infected at the examination 4 weeks previously.

Table 3. Feulgen reaction at different cell count intervals, grouped after mastitis status.

	Diamond fuchsin		Basic fuchsin		Total		Index
	no.	average reaction	no.	average reaction	no.	average reaction	
Cell count interval: 200—500,000							
A	127	1.28	81	2.07	208	1.59	100
B	6	2.58	9	3.18	15	2.94	173
C	12	2.00	13	2.77	25	2.40	145
D	8	3.25	8	3.96	16	3.60	222
Cell count interval: 500,000—1 mio							
A	48	2.08	14	3.74	62	2.46	100
B	12	3.08	5	6.30	17	3.95	152
C	28	3.08	34	3.82	62	3.49	123
D	8	3.44	1	7.00	9	3.83	167
Cell count interval: 1—2 mio							
A	44	3.60	7	5.65	51	3.92	100
B	6	5.25	6	7.74	12	6.50	142
C	36	4.46	23	7.14	59	5.50	125
D	5	6.50	10	8.65	15	7.93	162
Cell count interval: 2—5 mio							
A	11	6.00	2	8.25	13	6.35	100
B	3	7.66	3	9.00	6	8.33	118
C	22	6.36	20	10.50	42	8.33	116
D	8	9.62	8	11.50	16	10.56	150

This distribution in groups shows that milk from quarters of group A containing physiological cell number regardless of cell level had the lowest Feulgen reaction.

Milk from quarters affected by mastitis of a duration of more than 4 weeks (group C) on an average showed the second lowest reaction, whereas milk from quarters of no reaction 4 weeks previously (group B) as well as the newly infected quarters in group D showed the highest Feulgen reaction in relation to the cell content.

If the degree of reaction at each individual cell level is given index 100 for group A, and the indices of the other Feulgen reactions are calculated in relation to the former, it is found that the relationship between indices of the 4 groups is of the same order of magnitude within the individual cell levels.

Examinations of the Feulgen reaction on cells separated from the milk by centrifugation

In Figs. 6 and 7 it appears, as mentioned above, from the lines of regression between log Feulgen reaction and log added DNA, that the Feulgen reactions by the examination of milk samples are higher than could be expected from the content of whole cells, provided that the DNA-quantity in an intact cell is 7×10^{-12} g. Theoretically, this can be explained in two ways. Either, the cells in the milk contain more than the quantity of DNA indicated, or, the milk sample contains other elements, which are responsible for a Feulgen reaction.

In order to study these conditions milk samples were centrifugated.

After centrifugation the skim milk fraction was removed, and cell-free milk in a quantity corresponding to that of the removed skim milk was added to the sediment and cream layer. Milk was considered cell-free, if the content of cells was lower than 100,000 cells per ml and without any demonstrable Feulgen reaction. By means of the microscope it was controlled that the skim milk fraction contained less than 50,000 cells per ml.

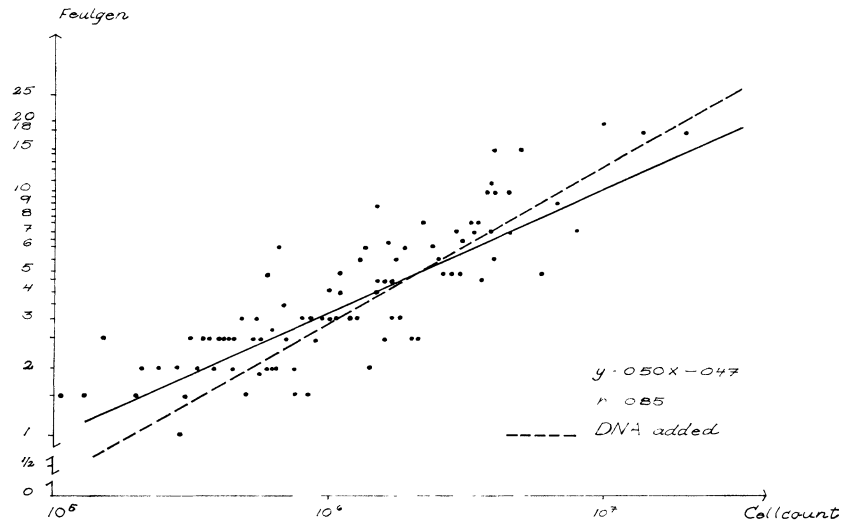
The correlating values of the cell content in milk, counted prior to centrifugation, and the Feulgen reaction on suspensions of sediment and cream layer in cell-free milk are shown in Fig. 9 A.

It is seen that the line of regression found on the basis of these results almost correlates with the one expected in theory. This proves that the content of DNA in the cells of the milk is within the order indicated.

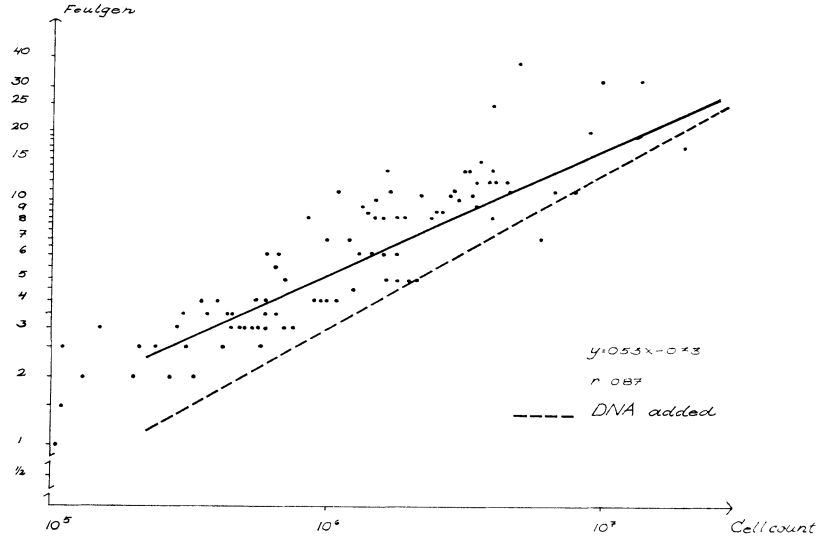
In Fig. 9 B the Feulgen reactions are recorded for the same milk samples before centrifugation. As in previous comparisons in Figs. 6 and 7 the results here are found to be higher than those expected in theory. The Feulgen reaction in milk therefore is reduced by replacing the skim milk fraction with milk of no cell content and Feulgen reaction. This suggests a content of reacting elements in the skim milk fraction.

Examinations of Feulgen reaction in cell-free skim milk were therefore carried out.

Cell counts and Feulgen reaction were performed on 80 milk samples. The results of these examinations are recorded in



F i g u r e 9 A. The relationship between log Feulgen reaction and log cell count. (The Feulgen reaction is determined on the milk samples after the skim milk has been replaced by cell-free milk).



F i g u r e 9 B. The relationship between log Feulgen reaction and log cell count before centrifugation. (Same milk samples as in Fig. 9 A).

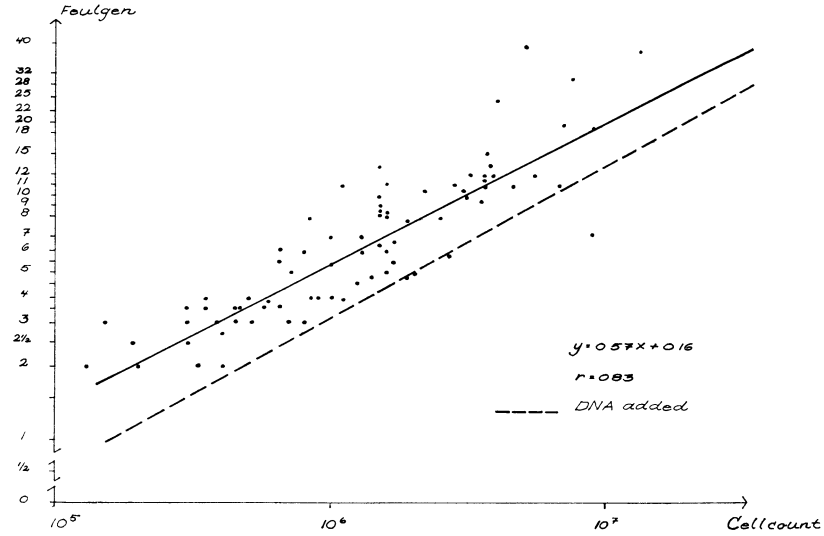


Figure 10A. The relationship between log Feulgen reaction and log cell count before centrifugation.

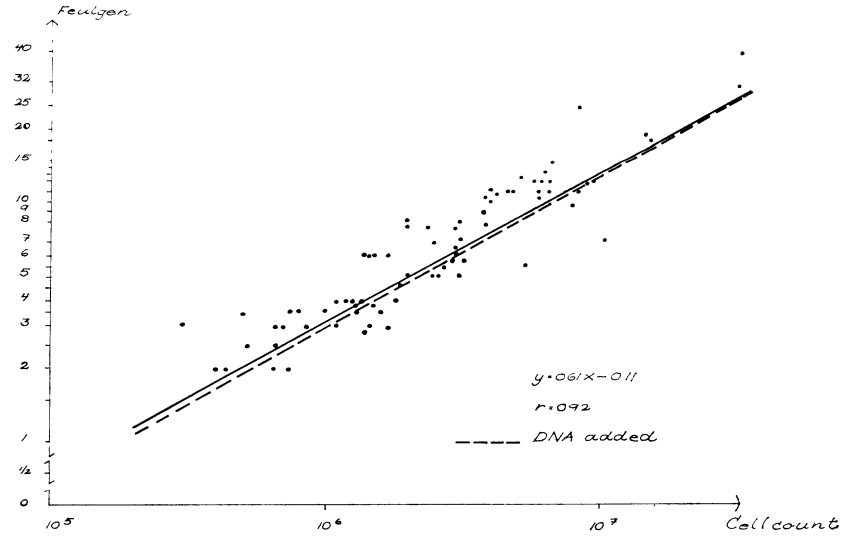


Figure 10B. The relationship between log Feulgen reaction and log cell count + the cell content, calculated on the basis of the Feulgen reaction in the skim milk fraction. (Same milk samples as in Fig. 10A).

Fig. 10 A. As in former examinations the Feulgen reactions are higher than anticipated according to the theoretical line of regression. After centrifugation of the milk samples Feulgen reaction was determined on the skim milk fraction; also, in the same way as described earlier, it was controlled that the cell content of the skim milk fraction was lower than 50,000 per ml. On the basis of the line of regression corresponding to the Schiff's reagent employed (see Fig. 5) it is possible to calculate the amount of DNA represented by the Feulgen reactions in the skim milk fraction. One µg of DNA corresponds, as previously mentioned, to 145,000 cells. It now becomes possible to calculate the dimensions of the cell content per ml, to which the Feulgen reactions correspond. In Fig. 10 B the ordinate represents the Feulgen reaction in the milk samples before centrifugation, and the abscissa represents the cell content of the milk sample based on the microscopic count + the cell content, which is calculated on the basis of the Feulgen reaction on the skim milk fraction. The regression between the logarithms to the abscissa and ordinate values shows, as it appears from Fig. 10 B, that the calculated line coincides with the theoretical line. It furthermore appears that the correlation, with the latter method, see Fig. 10 B, is better ($r = 0.92$) than with the former method, see Fig. 10 A ($r = 0.83$). This supports the fact that the method, employed in the determination of DNA in milk samples, is most applicable (see Fig. 4); it also proves, however, that the correlation between the cell content and the Feulgen reaction is reduced due to reacting elements present along with the number of countable whole cells.

DISCUSSION

Accuracy of analysis

The correction at 485 nm for different background absorption of reaction mixture and blank has the advantage of reducing the standard deviations, which again means an increase of the accuracy, with which the Feulgen reaction can be determined in milk samples.

The standard deviations found are lower in absolute value, but expressed in percentage they are higher the lower the Feulgen reaction is measured to be. The situation is the same by the direct microscopic cell count (Fig. 3).

In comparing the standard deviations for direct microscopic cell counts and Feulgen reactions it is found that the accuracy of analysis of the Feulgen reactions is of the same order as by direct microscopic cell counts using a working factor of 17,000.

Specificity

By addition of deoxyribonucleic acid to milk a Feulgen reaction is obtained. This is done by addition of a few µg of DNA per ml (Fig. 4). Determinations of quantities below 5 µg of DNA per ml are inaccurate. *Hauke* (1967) was also able to demonstrate the addition of 5 µg of DNA. These examinations must have rendered it probable that the Feulgen reaction is of much higher accuracy than has been indicated by *Widstrom* (1928).

The basis of the Feulgen reaction is a reaction between 2-deoxyribose and Schiff's reagent. 2-deoxyribose is produced by hydrolysis of the deoxyribonucleic acid. Thus, it is not DNA that can be demonstrated. The perishing cells, from which DNA remains sufficiently intact for the production of 2-deoxyribose by hydrolysis, therefore also give Feulgen reaction.

On an average the Feulgen reactions were smaller (Table 3), if the milk originated from quarters of physiological cell content, than if milk samples came from quarters suffering from mastitis.

In milk samples from quarters suffering from mastitis the highest Feulgen reactions were determined at fresh infections (less than 4 weeks old). These factors apply whether the agent can be demonstrated or not.

The author is of the opinion that these differences can be explained by the fact that in quarters, in which the cell content in the milk is physiological, it is a case of mere desquamation and excretion of cells.

In milk from quarters affected by mastitis the excreted cells serve to destroy the infecting agent, which leads to destruction of a certain number of cells on account of the effect of the agent. If the infection has been present for a longer period, a certain balance has been reached between bacteria and organism in the way that the destruction of the cells becomes moderate. In quarters presenting fresh infections the struggle between agent and organism is more intensive with subsequent destruction of a higher number of leucocytes and also of epithelial cells which are lysed by bacterial toxins.

By centrifugation of milk samples of a high cell content *Hauke* finds that an essential part of the Feulgen reaction originates from the skim milk fraction. *Paape & Tucker* (1966) find variations in the size of the Feulgen reaction to be related to the cell number in milk taken at different times during milking.

Hauke as well as *Paape & Tucker* express the theory that the cause of such variations are reactions from destroyed cells. As indicated above, this theory must be considered the most probable. If this theory can be confirmed in continued studies, it means that the Feulgen reaction is a more accurate measure for the total cell content of the milk than is the cell count, whether this is determined microscopically or electronically. On the basis of this theory a cell count can only be an estimate of the number of cells, which at an accidental moment of analysis happens to be undamaged and intact.

Correlation to contents of cells and DNA

The coefficient of correlation between the amount of DNA, which has been added to a cell-free milk sample, and the Feulgen reaction is extremely high. In the present study the coefficient of correlation was 0.98. In the 10 portions of Schiff's reagent, prepared at different days, the coefficient of correlation ranged from 0.97 to 0.99 (see Fig. 5). This suggests that the method employed for these tests for measuring the Feulgen reaction is most applicable for the determination of the DNA-content in milk.

The coefficient of correlation between the cell content of the milk and the Feulgen reaction is dependent on the accuracy with which each of the two methods of analysis is performed. If the cell number and Feulgen reaction are determined with the accuracy which constitutes the basis of the results listed in Fig. 6, and the milk samples analysed in the test are prepared as a mixture of milk of different origin, then $r = 0.98$.

If the Feulgen reaction is determined by one single examination of each milk sample, and the cell number estimated at a working factor of 20,000, and the milk samples are quarter milk samples from cows at varying stage of mastitis, then $r = 0.83$.

The coefficient of correlation between the actual cell content of the milk samples and the Feulgen reaction determined at a single measuring must be found between the above two values, probably around 0.90.

Coefficients of correlation of 0.909, 0.98 and 0.85 found by *Paape et al.* (1964), by *Hauke & Lüttich* (1966) as well as by *Hauke* appear to confirm the above observations.

CONCLUSION

The examinations have proved that the Feulgen reaction is an accurate method for the determination of the DNA-content in milk. The method is equally very applicable for determination of the cell content in milk, but the correlation is not so strong as in the determination of the DNA-content.

The accuracy of the method is of the same order as the direct microscopic cell count method at a working factor of 17,000 for determination of the cell content.

The Feulgen reaction was found to be higher, on the same content of cells, in foremilk samples from quarters affected by mastitis, than on equivalent milk of physiological cell content.

The theory is advanced that this difference is explained by the fact that damaged cells, not countable microscopically, give Feulgen reaction. Further studies are required to ascertain whether the higher Feulgen reaction in milk from mastitis-affected cows is a correct measurement for destroyed cells. Furthermore it must be examined which of the two, the Feulgen reaction or the cell count, best illustrates changes in the chemical composition of the milk.

Studies for clarification of these factors have been initiated.

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SUMMARY

The Feulgen reaction is examined by absorption photometric measurements at 565 nm with correction for varying background absorption at 485 nm. This correction is done to improve the accuracy of measuring.

Examinations of the accuracy of analysis for the Feulgen reaction and the direct microscopic cell counts show that the former is of the same order, when the cell count is made at a working factor of 17,000.

The standard deviations expressed in percentage of the reaction value are greater the lower the reaction is, whereas the absolute standard deviation is reduced.

It has been demonstrated that addition of a few (< 5) µg of DNA per ml milk can give Feulgen reaction. The coefficient of correlation between log added amount of DNA and log Feulgen reaction is 0.98.

The coefficient of correlation between log Feulgen reaction and log cell content depends on the accuracy at which both determinations are made. In a determination of cell content at a working factor of 550, and several determinations of the Feulgen reaction the coefficient of correlation (r) is found to be 0.98. A cell content determination at a working factor of 20,000 and a single determination of Feulgen reaction on each milk sample yields $r = 0.83$.

An examination of foremilk samples of the same cell content reveals on an average the highest reaction in mastitis-affected quarters which have been infected within the last 4 weeks. Quarters that have been infected for more than 4 weeks, on an average show the second highest reaction, whereas quarters of physiological cell number show the lowest reaction.

The DNA-content in most cases is found to be too high compared

with the number of cells counted microscopically. The DNA-content in centrifugated cells corresponds to the one calculated theoretically. The surplus DNA-content can be demonstrated in the cell-free skim milk fraction, probably originating from destroyed cells.

The studies performed suggest that determination of the content of 2-deoxyribose in milk by means of the Feulgen reaction is a more correct measure of the cell content in the milk than is the microscopic cell count. Studies are being continued for illustration of these conditions.

ZUSAMMENFASSUNG

Absorptionspektrophotometrische Bestimmung von DNS (Desoxyribonukleinsäure) in Milch ad modum Feulgen.

Die Feulgenreaktion ist durch absorptionsphotometrische Messungen bei 565 nm mit Korrektion für verschiedene Hintergrundabsorption bei 485 nm untersucht worden. Durch diese Korrektion ist die Genauigkeit der Messungen erhöht.

Die Untersuchungen der Analysengenauigkeit bei der Feulgenreaktion und der direkten mikroskopischen Zellzählung zeigen, dass diese von derselben Grössenordnung sind, wenn die Zellzählung bei einem Arbeitsfaktor von 17000 vorgenommen wird.

Ausgedrückt in Prozent der Reaktionswerte wird die Streuung grösser je kleiner die Reaktion ist, während die absolutte Streuung kleiner wird.

Es ist nachgewiesen worden, dass ein Zusatz von ganz wenig (< 5) µg DNS pro ml Milch eine Feulgenreaktion hervorrufen kann. Der Korrelationskoeffizient zwischen log zugesetzter Menge DNS und log Feulgenreaktion ist 0,98.

Der Korrelationskoeffizient zwischen log Feulgenreaktion und log Zellzahl hängt von der Genauigkeit ab, mit welcher die Bestimmung vorgenommen wird. Bei Zellzahlbestimmungen mit einem Arbeitsfaktor von 550 und bei mehrere Bestimmungen der Feulgenreaktion ist der Korrelationskoeffizient (r) = 0,98. Bei Zellzahlbestimmungen mit dem Arbeitsfaktor 20000 und einer einzelnen Bestimmung der Feulgenreaktion in jeder Milchprobe ist r = 0.83.

Bei Untersuchungen von Vormelk Viertelproben wird bei derselben Zellzahl durchschnittlich die höchste Reaktion in von Mastitis angegriffenen Vierteln gefunden, wo der Angriff innerhalb der letzten vier Wochen stattgefunden hat. Durchschnittlich zeigen Vierteln, die länger als vier Wochen angegriffen gewesen sind, die zweithöchste Reaktion, während Vierteln mit physiologischer Zellzahl die kleinsten Reaktionen aufweisen.

Der DNS-Gehalt zeigt sich in den meisten Fällen zu gross im Verhältnis zu den mikroskopisch gezählten Zellen. Der DNS-Gehalt in den freizentrifugierten Zellen ist wie theoretisch berechnet. Die überschüssige DNS-Menge kann in der zellenfreien Magermilchfraktion nachgewiesen werden und entstammt wahrscheinlich zerfallenen Zellen.

Die vorgenommenen Untersuchungen indizieren, dass eine Bestimmung des Gehaltes an 2-Desoxyribose in der Milch mit Hilfe der Feulgenreaktion ein korrekteres Mass für den Zellgehalt in der Milch ist als die mikroskopische Zellzählung. Weitere Untersuchungen zur Beleuchtung dieses Verhältnisses sind im Gange.

SAMMENDRAG

Absorptionsspektrofotometrisk bestemmelse af DNA (desoksyribonukleinsyre) i mælk ad modum Feulgen.

Feulgenreaktionen er undersøgt ved absorptionsfotometriske målinger ved 565 nm med korrektion for forskellig baggrundsabsorption ved 485 nm. Ved denne korrektion opnås en forøgelse af målenøjagtigheden.

Undersøgelser af analysenøjagtigheden for Feulgenreaktionen og direkte mikroskopisk celletælling viser, at denne er af samme størrelsesorden, når celletællingen foretages ved en arbejdsfaktor på 17.000.

Standardafvigelsen udtrykt i procent af reaktionsværdien bliver større jo mindre reaktion, medens den absolutte standardafvigelse bliver mindre.

Det er påvist, at tilsætning af nogle få (< 5) µg DNA pr. ml mælk kan give Feulgenreaktion. Korrelationskoefficienten mellem log tilsat mængde af DNA og log Feulgenreaktion er 0,98.

Korrelationskoefficienten mellem log Feulgenreaktion og log celletal afhænger af den nøjagtighed, hvormed begge bestemmelser foretages. Ved celletalsbestemmelse med arbejdsfaktor 550, og ved flere bestemmelser af Feulgenreaktionen findes en korrelationskoefficient (r) på 0,98. Ved celletalsbestemmelse med arbejdsfaktor 20.000 og en enkelt bestemmelse af Feulgenreaktionen på hver mælkeprøve findes $r = 0{,}83$.

Ved undersøgelse af formælksprøver findes ved samme celleindhold gennemsnitlig den højeste reaktion i mastitisangrebne kirtler, hvor angrebet er sket indenfor de sidste 4 uger. Gennemsnitlig viser kirtler, der har været angrebet i længere tid end 4 uger, den næsthøjeste reaktion, medens kirtler med fysiologisk celletal har de mindste reaktioner.

DNA-indholdet findes i de fleste tilfælde for stort i forhold til mikroskopisk talte celler. DNA-indholdet i fracentrifugerede celler er som teoretisk beregnet. Den overskydende DNA-mængde kan påvises i den cellefri skummetmælksfraktion, formentlig hidrørende fra henfaldne celler.

De foretagne undersøgelser indicerer, at bestemmelse af mælkens indhold af 2-desoxyribose ved Feulgenreaktion er et mere korrekt mål for mælkens celleindhold end den mikroskopiske celletælling. Fortsatte undersøgelser til belysning af dette forhold pågår.

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