AN EVALUATION OF THE RESAZURIN TEST IN MILK SANITATION

by

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TABLE OF CONTENTS

I. INTRODUCTION

The reductase test, with methylene blue as an indicator, has been in use for over thirty years in determining the bacteriological quality of raw milk. During that time, particularly after its adoption as a standard method of milk analysis by the American Public Health Association, various defects of the methylene blue test have become apparent, and other dyes have been sought which would eliminate some of these objections.

With this purpose in mind, the dye resazurin ($C_{12}H_7O_4N$) was introduced to replace methylene blue in the reductase test. At present, a considerable amount of experimental data has accumulated on the resazurin reductase test. Many of the reports claim that resazurin is a more efficient and reliable indicator of raw milk quality than is methylene blue. The 8th edition of Standard Methods for the Examination of Dairy Products mentions the resazurin test, but it has not been accepted as a standard method.(2) This publication states:(*)

> The chemistry of resazurin, the suitability of the commercial preparations now on the market, end points, anomalies, and a suitable resazurin test are all at present in controversy. In view of this situation, no recommendation either for or against the use of this test is made at the present time.

Although a number of these points have been clarified since the above was published, (1941), it was found in reviewing the literature

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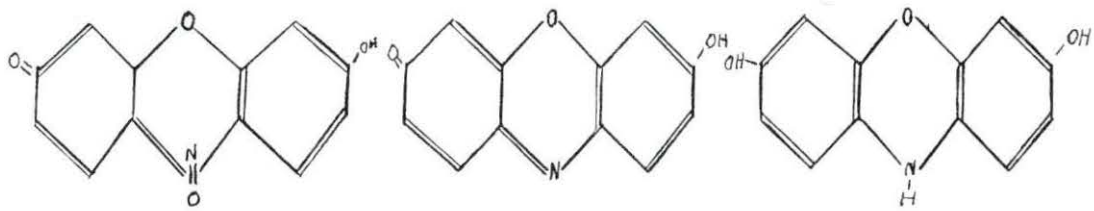
(*) American Public Health Association. Standard Methods for the examination of dairy products. 8th ed. P.66. New York. 1941.

that there is little agreement as to whether the resazurin test is a suitable test of milk quality. This suggested a study to decide on a suitable resazurin test, and, after comparing it with other standard tests to evaluate its usefulness to the milk sanitarian in routine control work, or in a quality improvement program. In addition, a test was sought which would indicate the presence of mastitis milk in bulk producers samples and thus point out the herds requiring mastitis control. The resazurin test for this purpose was compared to the commonly used mastitis tests, physical examination of the udder, and inspection of the dairy farm premises.

The abbreviation "Standard Methods" which appears in this thesis, refers to Standard Methods for the Examination of Dairy Products - Microbiological, Bioassay and Chemical, and is used to avoid repetition of this cumbersome title.

II. REVIEW OF LITERATURE

Pesch and Simmert (39) in 1928 appear to have first used resazurin in testing milk, and they were followed shortly afterward by Waldbauer (56). Mundinger and Wolf (34) in 1933 described the complete reduction of resazurin as occurring in two steps. The first step, in which resazurin changed to resorufin, was said to be irreversible. In the second step the resorufin changed to hydroresorufin, a reversible reaction.



Resazurin (blue) | Resorufin (pink) | Hydroresorufin (colorless)

The end product was later designated as dihydroresorufin by Baker (4) and by Twigg (55).

Ramsdell, et al (44) in 1935 published the first comprehensive work on the resazurin test, and interest in the test as a practical measure dates chiefly from this publication. Ramsdell's study showed that when the test was applied to abnormal milk, a characteristic reaction resulted--an early initial reaction followed by a delayed final reaction. Their results showed a general relation between the leucocyte content and resazurin reduction, and was the first work suggesting that the resazurin test could be influenced by cells other than bacterial. They were unable to produce resazurin reduction by adding washed leucocytes to milk. Ramsdell

reported that milk with a high catalase reading reduced resazurin within one hour, hence he suggested that only one hour was required for the test, a contention which has formed the basis for much of the controversy surrounding it. The chief conditions reflected by the resazurin test in milk were outlined as follows;(*)

1. Rapid reduction, quickly completed.

a. High contamination with actively growing bacteria.
b. Presence of strongly reducing organisms.
c. Pathologically abnormal milk, i.e. mastitis milk.
d. Physiologically abnormal milk, colostrum or late lactation.

2. Slow reduction during 4-6 hours of incubation.

a. Low bacterial content.
b. High content of dead, inactive or weakly reducing organisms.
c. Low cell content.

3. Slow reduction, followed by rapid reduction.

a. Physiologically normal milk with a low bacterial content.
b. High bacterial count with organisms in lag phase at first. Reduction becomes rapid as the logarithmic phase is entered.

4. Rapid reduction, followed by slow reduction.

a. Pathologically or physiologically abnormal milk.
b. High bacterial content, but bacteria well advanced in the logarithmic phase, and hence poor reducers.

Strynadka and Thornton (49) in 1938 added washed leucocytes to normal milk and were unable to demonstrate any effect on methylene blue reduction. They concluded that the abnormal udder conditions responsible for abnormal cell content are responsible for abnormally high concentrations of reducing substances in milk. They did not identify these

(*) Ramsdell, G.A. et al. Investigation of resazurin as an indicator of the sanitary condition of milk. Jour. Dairy Sci. 18;715. 1935.

substances but merely inferred their presence.

Little (31) also studied the effects of leucocytes in milk on resazurin reduction. He found that the dye was very sensitive to pathologically or physiologically abnormal milk, as indicated by a high cell content or mastitis streptococci, or both; whereas the methylene blue test was only slightly affected by these factors. He concluded that these factors account for much of the disagreement between the two tests in milks which were slow on methylene blue reduction. This was later confirmed by Barkworth (5), and by Thomas and Probert (53). Johns and Howson (28) pointed out another source of disagreement, viz., that resazurin was much more sensitive than methylene blue to weakly reducing organisms.

Barret et al (7) found that staphylococci and cells due to mastitis or colostrum caused rapid reduction of resazurin, making the test very sensitive to abnormal milk. They claimed that the resazurin test gave as much information in one hour as the methylene blue test in seven hours. This claim was subsequently repeated by Warner (57). Other investigators, however, made more conservative claims, stating that resazurin gave as much information in one hour as methylene blue in six hours (30), in five hours (43), (8), or in two hours (54).

Warner (57) compared resazurin with methylene blue and found no significant difference between the reduction time to white of either dye. He was unable to show any relation between resazurin pink and the leucocyte count or standard plate count. He first reported the effect of light on the resazurin test, and found that indirect light was of no practical

significance. Direct light, however, so speeded up the reduction that many samples were seriously downgraded. This work was confirmed in 1938 by Frayer (17), who found that resazurin was very sensitive to sunlight and artificial light. However, Thornton et al (54) stated in 1941, that they had not observed this sensitivity which others had reported.

Warner found that the reduction time for a sample of homogenized raw milk was shorter than that for a sample of the same milk that was not homogenized. He plated the samples and found a higher plate count in most of the homogenized samples. He concluded that both the higher plate count and the shortened resazurin reduction time were due to the breaking up of the bacterial clumps in the homogenized sample. However, Johns (24) set forth that the clumping of organisms had little or no effect on reduction time. Warner's second suggestion, that the faster reduction in homogenized milk was due to the fat globules not rising, thereby preventing a large number of bacteria from being swept out of the milk-dye mixture, was borne out by later work.

Frayer (17) reported in 1938 that the resazurin test showed little correlation with the standard plate count; however, this work was done before the adoption of the T.G.E.M. agar prescribed by the 8th edition of "Standard Methods". He found it showed a fair correlation with the cell content of milk drawn aseptically from individual cows; that it was sensitive to the presence and activity of bacteria; that efficient initial cooling seemed to retard reduction; and that replicate tests yielded similar results, making the test a dependable one. The latter point was con-

firmed by Phillips and Soulides in 1943 (40).

Using the .005 per cent solution of the dye, Frayer found that the test was only slightly affected by minor variations in dye concentration. This solution was suggested by Moldavan (32), and has been widely adopted, since minor errors in measuring are of less significance in 1 ml. of a .005 per cent solution than in .1 ml. of the .05 per cent solution suggested by Ramsdell. This portion of Frayer's work was confirmed in 1939 by Davis & Thiel (13). Fabian, however, suggested the use of the .05 per cent solution as late as 1945 (16).

Frayer found that initial cooling and holding seemed definitely to retard reduction. This effect had been previously noted by those working with the methylene blue test. Frayer referred to Johns' hypothesis (23) that prolonged reduction time with methylene blue was due to the fact that the bacteria were rendered dormant by prolonged refrigeration and hence took longer to attain their maximum growth than those not refrigerated. However, Frayer felt that in the case of resazurin, a dye more sensitive to exhaustion of the oxygen dissolved in the milk, low temperature had a more direct effect, viz., the milk contained more dissolved oxygen, and the bacteria and cells consequently took longer to use it up. He based this contention on the observation of Jackson (22), that milk drawn anaerobically from the udder reduces methylene blue almost instantaneously, and the known fact that water can dissolve nearly twice as much gas at 40°F as at 98°F, and milk could be expected to behave in the same way. In presenting this hypothesis he did not attempt to refute the point that low temperature retarded cell metabolism, but merely to ela-

borate on it.

Apparently in conflict with Frayer's observations, Morris and Edwards found that storage of milk at 40°F for 18 hours prior to testing appeared to accelerate the reduction of milk with a high cell content (35).

Nelson and Foltz (37) in 1938 found that milk with a leucocyte count above 500,000 tended to produce a change in resazurin within one hour. They also reported that a number of the commercial dye preparations were useless for testing purposes.

Nelson (36) and other workers confirmed the fact that various batches of resazurin dye varied greatly, and emphasized the need for a reliable dye suitable for testing purposes.

In 1939 Johns (24) outlined the advantages of the resazurin test over counting methods, on the basis of his work:

> 1. Examines a much larger portion of the milk than counting methods, thus avoiding serious errors frequently met with in such methods due to uneven distribution of the organisms in the milk.
>
> 2. The clumping of organisms has little or no influence on the reduction time, while the plate count, and to a lesser extent the direct microscopic count, are seriously affected.
>
> 3. The reduction test is sensitive to the state of activity of the organisms which the counting methods fail to reflect.

Johns pointed out the necessity of inverting the tubes every thirty minutes during incubation to keep the bacteria uniformly distributed throughout the milk. This procedure gave a truer picture of the oxygen consuming activity of the bacterial flora. Subsequent investigation by Davis and Thiel (13) confirmed these results, and it is now generally accepted that the

tubes should be inverted at intervals during incubation. Skar (48) recommended that the tubes be filled to the top to exclude air, and a glass bead placed in each tube to aid mixing upon inversion. Abele (1) reported that inverting the tubes every 30 minutes in the methylene blue test gave a higher degree of agreement between plate count and reduction time than did the "Standard Methods" (8th ed.) test.

Johns (24) thought that recording the resazurin color after one hour as recommended by Ramsdell (44) was less accurate than recording the time taken to reach the pink stage, as a high proportion of high-count milks were placed in the highest grade by the first method. For this reason Johns favored the latter method of reading the test. However, Davis (10), while admitting the truth of this contention, did not agree with his recommendation. He pointed out that one of the advantages of the resazurin test over the methylene blue was that it yielded information in $\frac{1}{2}$-1 hour instead of 4-5 or more hours. By waiting to read the test when it reached the pink stage, this advantage would not be utilized.

Johns also observed that the correlation between resazurin color and cell count was poorer with market milks than with quarter samples. This he attributed to the fact that cells would be fresh and unwashed in quarter samples, whereas they would tend to be aged and washed (diluted with other milk) in producers bulk samples.

Davis and Thiel (13) in 1939 compared: (a) Observing the time required for reduction to an arbitrarily defined end-point, with (b) recording the color after a given time, e.g. $\frac{1}{2}$ or 1 hour. They found that both methods gave much the same results for observation times up to two hours.

They reported 22 per cent contradictions between the resazurin test and the plate count, and 10 per cent between resazurin and methylene blue. Three per cent of their samples were unjustly condemned by resazurin, i.e. failed resazurin and passed the plate count. They stated the latter were abnormal milks because they were catalase positive. However, it should not be inferred from this that the discrepancy was due solely to the reducing activity of body cells. Packer (38) reported that micrococci grew sufficiently in milk in 24 hours to produce a positive catalase test.

Davis and Thiel as well as numerous other workers suggested various colors at a specified time as being indicative of certain grades of milk quality; but as these colors did not refer to any definite standard, they are not quoted here. Johns and Howson (29) suggested the use of the Munsell color system to provide a standard when they published their work on the triple-reading test in 1940. They also emphasized the importance of a standard source of light in reading the test. A color comparator for resazurin testing was devised by Davis and Thomas in 1940 (14). They adapted it from the Lovibond comparator used for reading the phosphatase test.

In 1939, Russel et al (67), proposed the resazurin test as a diagnostic test for mastitis. Quarter samples were taken from subclinical cases during the middle of lactation. With cultural examination and cell count as the basis of comparison, they reported getting 1.8 per cent false positives, these cases being negative to the first two tests. The percentage of false negatives, in which resazurin failed to detect cases diagnosed by the other two tests was much higher, in the neighborhood

of 25 per cent. These workers reported that the resazurin test was sensitive to the presence of streptococci and staphylococci but not to micrococci. They pointed this out as a valuable feature, since micrococci seemed rarely to be involved in the etiology of mastitis. They also remarked that since the color changes in resazurin were due to both reductase and catalase, its results were more accurate than those of the pH indicators, such as brom thymol blue.

Davis (9) described the resazurin-rennet test in 1939. He proposed it for the sanitary control of raw milk, and claimed that it fulfilled a "crying need". In this combined test, the resazurin indicated the cellular and bacterial content; and the rennet, the chemical composition of the milk. Davis standardized his rennet to clot normal milk in less than 60 and more than 30 minutes, and then classfied milk on the basis of clotting time as follows:(*)

Time	Description	Reasons
clotted in $\frac{1}{2}$ hr	fast	acidity resulting from bacterial growth, or presence of colostrum
clotted in $>\frac{1}{2}$ hr but <1 hr	normal	
not clotted in 1 hr	slow	abnormal composition, low casein and calcium, high pH, globulin and chloride content

(*) Davis, J.G. The resazurin-rennet test for the commercial control of milk supplies. Dairy Industries. 4:422. 1939.

Davis classified milk on the basis of resazurin reduction as follows:

No change (blue) after 1 hour	-	good
No change (blue) after ½ hour, but changed after 1 hour	-	indifferent
Change to mauve, pink or white in ½ hour	-	bad

Schacht and Nichols (47) in 1941 reported using the resazurin-rennet test on producers bulk samples. They read the results at once, at the end of one hour of incubation, and classified milk according to Davis' classification, as outlined above. They did not attempt to classify color changes; the milk either remained blue or showed a change. On this basis, and using Davis' clotting times, they classed 36.4 per cent of their samples as unsatisfactory because of dye reduction, failure to clot, or both. Since methylene blue and Breed counts, which they ran simultaneously, showed much smaller percentages of poor quality milk, the question was raised whether the resazurin-rennet test condemned satisfactory milk. Schacht and Nichols attempted to evaluate the test by comparing it with sanitary conditions of the herd and farm instead of with commonly used standard tests. They concluded that the resazurin-rennet test showed unsatisfactory farm conditions in 89 per cent of cases, whereas methylene blue picked out only 14 per cent, and the Breed count 50 per cent.

In 1942, Davis (11) proposed the resazurin-rennet test for the detection of mastitis. He stated that mastitis samples usually reduced the dye and failed to clot. He did not propose this test as a substitute for plat-

ing, but claimed it to be the most informative of all the indirect tests, and one easily carried out. He found that resazurin did not affect the clotting phenomenon, nor did clotting affect reduction. The following is Davis' interpretation of the resazurin-rennet test after one hour of incubation, as used for the detection of mastitis:(*)

Resazurin	Rennet	Reading
blue	+	normal
mauve or pink	+	high in cells, but normal in composition
blue	-	normal in cells, but abnormal in composition
mauve or pink	-	high in cells and abnormal in composition

Morgan and M'Kenzie (33) took mixed fore-milk samples of individual cows and also bulk herd samples. They subjected the individual samples to various mastitis tests and both types of samples to the 1 hour resazurin test. They reported that the reducing activity of body cells which remained 24 hours after milking would rarely be sufficient to degrade the bulk milk of a herd. Thomas and Probert (53) stated that in 24 hour old milk a cell count of 1,000,000/ml. was required for active reduction.

Thomas and Bowie (51) in 1943 suggested caution in degrading milk with the resazurin test during the winter months, when a high proportion of the cows were in late lactation.

Davis et al (15) studied the part played by mastitis cells in high

(*) Davis, J.G. Bacteriological methods for the large scale detection of mastitis. Veterinary Record. 54:146. 1942.

count milk. Using the rapid resazurin test, they reported that 24 hour old cells did not appreciably influence the reduction.

Frayer (18) in 1940 described the role of the dissolved oxygen, nitrogen and carbon dioxide in milk on dye reduction, and elucidated the fundamentals of the resazurin reduction process. This was followed by a further report in 1942 in which he discussed critically the biochemical basis of dye reduction in milk. He pointed out that it would not be safely assumed "that all resazurin color changes in milk result from milk abnormalities deleterious to quality". (*)

Thomas (50) in 1943 described sterility investigations on resazurin solutions prepared from powder and tablets. He stated that solutions prepared from powder were less likely to have a high bacterial content than those prepared from tablets, as the "binder" used in the tablet acted as a bacterial nutrient.

Thomas found that removal of a portion of the cells from a milk sample by centrifuging and discarding the sediment resulted in a loss of reducing activity in the supernatant portion. He also found that by adding the centrifuge sediment to normal milk an increase in reduction was obtained. He compared the reducing activity of leucocytes in bulk producers milk and in samples of the same milk held for 24 hours either in the refrigerator or at atmospheric temperature. He found a significant decrease in reducing ability after 24 hours, even in individual cow samples.

Johns reported in a private communication to Davis (10) that the

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(*) Frayer, J.M. Dye reduction in milk related to Eh, pH and dissolved gases. Vermont Agr. Expt. Sta. Bull. 498:32. 1942.

thermoduric types of bacteria, when present in high numbers, behaved similarly to body cells in resazurin. They produced a slight color change fairly soon, then levelled off, and required 6 hours or more to reduce to white. This observation first pointed out the limitations of the resazurin test for pasteurized milk, although Warner's earlier work (57) suggested this possibility.

Provan and Rowlands (42) in 1943 suggested that resazurin might be more useful than plating methods in pasteurized milk control. Since the phosphatase test assayed the efficiency of the pasteurization process, there was needed chiefly a simple test to detect subsequent contamination. They criticized plating methods because these emphasized bacterial numbers, which in pasteurized milk were usually due to thermoduric organisms, which had little relation to the keeping quality. They pointed out that it was the organisms which survived in small numbers or were contaminants from plant equipment which influenced keeping quality; and that a suitable test was one which would reflect their presence, rather than mere numbers.

Rowlands (45) in 1944 described the application of the resazurin test to pasteurized milk control. The milk had to be stored at 60-65°F for a period before testing, to enable any contaminating bacteria from plant equipment to proliferate. Rowlands claimed that no unfairness would result to the plant operator from factors beyond his control, since the thermoduric bacteria were very inactive dye reducers. In other words, the test would not be influenced unduly by organisms contributed by the producer and surviving pasteurization.

In 1944 Johns (27) recommended the adoption of standard dye tablets which he described. These were approved that same year by the Biological Stain Commission, and a uniform source of dye was available for the first time to American workers, making it possible to compare results obtained in different laboratories.

Watts and Stirling (58) in 1944 carried out a careful study on the diagnosis of mastitis by the resazurin test, and on the influence of mastitis milk when mixed with normal milk during ordinary dairy operations. They used milk of high bacterial quality so that the effects observed were primarily attributable to body cell activity.

After selecting a standard, (Lovibond disc 4 within 30 minutes), they found false negative readings occurred in 88 per cent of their tests on quarter samples; and that no combination of color and incubation period would give less than 30 per cent error. They concluded that since failure to reduce resazurin was no indication of freedom from mastitis, that the test was of very limited value for its routine diagnosis.

Regarding the effect of bulking, they reported that individual cow samples showed a marked decrease in reducing ability compared to abnormal quarter samples; and that this decrease was correspondingly greater in milk-can samples containing several cows' milk. They noted that up to 73% of the milk in a can could be derived from cows infected with mastitis without significantly affecting the resazurin test.

Barkworth and Jones (6) in 1944 found there was a tendency among certain workers to record most samples as being a match to the comparator colors rather than attempt to interpolate. They concluded that proper care and discrimination were not always in use and recommended tightening

up of color matching requirements.

Davis and Jones (12) described attempts to obtain an inhibitor for leucocytes which would allow resazurin to reflect bacterial activity only. They were unable to arrive at a satisfactory method for inhibiting cell reduction.

Golding and Jorgensen (21) in 1945 compared the resazurin grade and the standard plate count for determining raw milk quality. In this study they used the T.G.E.M. agar prescribed by the 8th edition of "Standard Methods". They reported a closer agreement between the two methods than had earlier workers who used the previously prescribed agar. They also published a comparison table for the Lovibond comparator and the Munsell grader, and drew attention to the fact that accurate readings were impossible without the use of a comparator. They reported that the ten minute test was of real value in rejecting very poor milk, but that greater refinement could be obtained by continuing incubation to 30 or 60 minutes.

In 1945 Thomas and fourteen collaborators (52) reported the results of an exhaustive survey embracing 525,000 milk samples subjected to the routine resazurin test in 32 laboratories. In the British test, the producers milk is sampled at the time of delivery to the plant, and then kept in the shade at atmospheric temperature approximately 24 hours from the time of production. The storage box contains thermometers which record the minimum and maximum temperatures, and from these the mean temperature for the storage period is calculated. This temperature is then applied to a compensating scale, which tells the period of test incubation required for the samples. The incubation periods vary from 150 minutes for a stor-

age temperature of 35°F and lower, to 5 minutes for a storage temperature of 65°F and over. Incubation is at 37.5°C. The color obtained after the required incubation period is then applied to another table and the grade of the milk is read. The grades are A, B and C.

Thomas et al found the routine resazurin test could give a better correlation with keeping quality than could titratable acidity. They reported that natural illumination through a north window was best for reading the test, and recommended a flourescent light for seasons when natural light was inadequate.

It is of interest to note that resazurin has been proposed for control work in the food industry as being applicable to comminuted meats, eggs and dried vegetables(3), (41).

III. METHOD OF PROCEDURE

A. Obtaining Milk Samples

The milk samples used in the 1 hour and 10 minute (rapid) resazurin tests were pint samples taken by the Milk Sanitation Department of the City of Ames between 15 March and 15 July for routine analysis. They were producers' samples representing mixed morning and evening milk and were handled throughout the sampling procedure as prescribed by "Standard Methods". Samples for resazurin testing were withdrawn from the bottles immediately after the samples for plating. The milk samples were then subjected to the specific gravity, acidity and sediment tests.

The mastitis milk samples were quarter samples drawn under aseptic precautions. The udder was cleaned with a cloth wrung out of warm water containing 200 p.p.m. of chlorine. This was followed by swabbing the teat with 70% ethyl alcohol, paying particular attention to the orifice. Several squirts were drawn into a strip cup and examined for abnormal constituents. Six ml. of the fore milk was then milked into a brom cresol purple tube, 15 ml. into a tube for the catalase test, and 15 ml. into a sterile bottle for resazurin testing and for the preparation of Breed smears. Catalase tests were set up within an hour from the time the samples were drawn. Brom cresol purple tubes were read, and then incubated for the Hotis test. The incubated samples were plated on blood agar for cultural examination. Samples for resazurin were placed under refrig-

eration within an hour from being drawn and were tested within 4 hours.

B. Preparation of the Dye Solutions

1. Resazurin solution

Eastman resazurin powder was used at the beginning of the experiment. For a time resazurin powder and tablets were used simultaneously to compare their action, and the tablet solution alone was used during the latter two-thirds of the experiment. The tablets (National Aniline), are certified by the Biological Stain Commission* for use in milk testing.

In preparing the solution, a bottle of distilled water was sterilized in the autoclave. The bottle was removed and the powder or tablet added to it immediately, i.e., while still hot. In the case of the tablets a sterile forceps was used. In the case of the powder, a .005 per cent solution was prepared. The tablets contained 11 mg. of dye, and following the directions given on the bottle, a .0055 per cent solution resulted when one tablet was added to 200 ml. of water. The bottle was agitated until the resazurin was completely dissolved, and the solution dispensed in 50 or 100 ml. amounts into sterile, brown glass, screw-capped bottles. These were not refrigerated, but kept in the dark at room temperature. Fresh solutions were made up weekly and any remaining after a week's time discarded.

One ml. samples of 7 day old resazurin tablet solution were plated

*Cert. No. NR_z4

on five occasions using T.G.E.M. agar. None of the samples showed any colonies after 48 hours incubation at 37°C. Although the manufacturers do not state that the tablets are sterile, it was found that the solution could be prepared free from contamination, and would remain so without the elaborate storage technics recommended by some investigators.

2. Resazurin-rennet solution

For the resazurin-rennet test Hansen's cheese rennet (liquid) was used. A "normal" mixed milk was obtained from the milk cooler in the Iowa State College dairy, representing the secretion of the first six cows milked. This milk fulfilled the definition of a "normal" milk designated by Davis (9) for this purpose, as a milk from cows free from mastitis, with a plate count of less than 100,000, the cows being in full lactation.

0.1, 0.15 and 0.2 per cent solutions of rennet were prepared by adding the rennet to 100 ml. flasks of sterile distilled water. One ml. of each solution was added to 10 ml. of the test milk in duplicate tubes, and the milk incubated in a 37.5°C water bath. Incubation was commenced within 30 minutes of obtaining the milk. It was found that the 0.2 per cent solution clotted in 30 minutes, the 0.15 per cent solution in 40 minutes and the 0.1 per cent solution in 45 minutes. This procedure was repeated on two succeeding days with milk from the same source, the clotting time for the two weaker solutions being prolonged about 5 minutes on the third occasion.

A resazurin-rennet solution was prepared in the above concentrations

by adding the rennet to the prepared and cooled solution. Coagulation times were found to be approximately the same as for the aqueous rennet solution, the times being read to the nearest 5 minutes. It was decided to use the 0.15 per cent solution (0.15 ml. rennet in 100 ml. resazurin solution) as it fulfilled the requirements of clotting in less than 60 minutes and more than 30, and would give a quicker reading than the 0.1 per cent solution.

The rennet was kept in the freezing chamber of the refrigerator in a brown glass, screw-capped bottle. It was returned there immediately after use. Resazurin-rennet solutions were prepared fresh for use each time immediately before testing, and remaining solution discarded, as rennet quickly loses its potency in dilute solutions.

C. Technic of the Tests

1. Reading the tests

For reading the tests, artificial lights in the room were extinguished, and natural light was used. A window which was not receiving direct sunlight was chosen, and at no time were the samples exposed to direct light either natural or artificial. By moving the samples along the table to and from the window an optimum illumination which brought the color contrast out most clearly could quickly be found.

The rack of tubes was removed from the incubator or water bath, and the Munsell grader* used to select the colors in the milk tubes. During

*Resazurin Color Grader, manufactured by the Munsell Color Co., Inc., Baltimore, Md.

the course of this study, a modified comparator (Figs. 4 and 5) was devised using the Munsell grader colors, which facilitated the reading of the test. The modified Munsell grader was found to speed up the color matching process, which is important where the tubes are to be returned for further incubation. Figs. 1-2, and 3, show the Lovibond Comparator and the Munsell color grader, respectively. Fig. 4 shows the modified Munsell grader. Fig. 5 shows the Munsell grader above, and the modified version below.

It was found that the use of the tablet solution resulted in a closer match between the colors in the milk and those on the grader than did the powder solution. No difference in reduction times was noted on the duplicate samples run to compare the two solutions.

The grades used to designate the milks were 1, 2, 3, 4, 5, 6, 7, 8 and W (white). The colored papers on the Munsell grader were arbitrarily designated 1, 3, 5 and 7 reading from blue to pink. The numbers 2, 4 and 6 were assigned to colors intermediate between those on the grader, and the number 8 for the color intermediate between 7 and W. Therefore, 2, 4, 6 and 8 represent the reader's estimate rather than a definite color notation.

2. Ten minute and one hour resazurin tests

These tests were applied to producers' milks secured by the City of Ames Milk Sanitation Department. Glass tubes of 17 ml. capacity were used. These were provided with rubber stoppers and sterilized in the autoclave. No more than ten tubes were set up in a rack, so that the difference in the time the dye was added would not be too great from the first tube to the last. Ten ml. of milk was pipetted into each tube. This was followed by

1 ml. of resazurin solution. Ten ml. pipettes were used, and no pipette was introduced into the solution more than once. The tubes were stoppered and inverted once to mix the dye and milk. About 3 ml. of air remained in each tube after it was stoppered.

The tubes were immediately placed in the 37°C incubator and read after 10 minutes and after 1 hour of incubation. Each rack was inverted once, half an hour after commencing incubation. Samples 78 to 85, and 96 to 122 were run in duplicate to compare the reduction obtained with resazurin solution prepared from powder (Eastman) and tablets (National Aniline).

3. Resazurin-rennet test

This test was applied to quarter samples from quarters giving positive readings on one or more of the mastitis tests. Sterile tubes were used as for the resazurin test. Ten ml. of mastitis milk was pipetted into each tube, followed by 1 ml. of resazurin-rennet solution. The tubes were stoppered and inverted once to mix the dye and milk, then placed in a 37.5°C water bath. Following this, duplicated Breed smears were prepared and counted, and the counts averaged. Readings were made every 15 minutes for resazurin color and for coagulation. A tube of normal milk, as defined under section B-2 above, was placed in each rack with the mastitis samples as a control.

During the study, fourteen of the mastitis milks were drawn in larger amounts, and placed in the rack in duplicate. One set of tubes received resazurin-rennet solution, and the duplicate set resazurin solution. Readings were made to compare the rates of reduction in the milk of the two

solutions. No difference in reduction rate was observed between the mastitis samples tested with resazurin and those tested with resazurin-rennet solution.

4. Effect of udder cells in sterile milk

Low count milk was obtained from the Iowa State College Dairy, tubed in 10 ml. amounts and "sterilized" in the autoclave. This milk was held 10 minutes at 221°F. While this did not result in absolute sterility, it enabled the milk to remain white, and thus give a normal color match with the addition of resazurin.

Freshly drawn mastitis samples were pipetted into sterile centrifuge tubes in 10 ml. amounts and centrifuged 20 minutes at speed no. 20. The result of this was that 60-70% of the body cells were deposited in the centrifuge sediment, as determined by Breed counts before and after centrifuging.

The butter layer on the centrifuged samples was then broken up with a sterile needle and the supernatant poured off into a sterile tube. The remaining sediment was resuspended in $\frac{1}{2}$ ml. of "sterile" milk, which was decanted back into the remaining $9\frac{1}{2}$ ml. in the tube. The tubes were then refrigerated for 1 hour to inhibit bacterial growth.

For testing, the samples were set up in the rack as follows: one tube of mastitis milk, one tube of the same milk with a portion of the cells centrifuged out, one tube containing the centrifuge sediment in sterile milk, and a tube of sterile milk as a control. One ml. of resazurin solution was added to each tube and they were incubated in a 37.5°C water

bath. Readings were made every 15 minutes for two hours and discontinued after that time, as it was felt that period was ample to show body cell activity, and further incubation would reflect the activity of bacterial growth.

IV. RESULTS

A. Ten Minute and One Hour Resazurin Tests

For purposes of comparison with the Standard Plate Count, both the resazurin grades and the plate counts have been placed into groups. Table 1 shows the number of samples of each plate count group falling into the paired resazurin groups for the one hour test.

Table 1

Relation Between Resazurin One Hour Test and Plate Count

Standard Plate Count	Resazurin Grades 1 & 2		3 & 4		5 & 6		7 & 8		W		Totals
	no.	%	no.	%	no.	%	no.	%	no.	%	
75,000 and under	83	60.5	46	33.5	8	6	-		-		137
76,000 - 100,000	4	30.8	7	53.8	1	7.7	1	7.7	-		13
110,000 - 250,000	10	38.4	14	54	1	3.8	1	3.8	-		26
260,000 - 500,000	13	27.6	22	46.8	9	19.2	3	6.4	-		47
510,000 - 1,000,000	11	24.4	16	35.6	12	26.7	5	11.1	1	2.2	45
1,100,000-5,000,000	2	2.4	15	18	16	19.3	33	39.8	17	20.5	83
5,100,000 and over	-		-		3	5.6	9	17	41	77.4	53
									Total Samples		404

The most clear cut results in Table 1 appear in the first and last plate count groups. Out of 137 samples with a plate count of 75,000 or

less none reduced the dye beyond grade 6. All of the 53 samples with a plate count of 5,100,000 and over reduced the dye to grade 5, 6, or beyond. Hence if a sample reduced to grade 7 in one hour it could be expected to have a plate count above 75,000, and if it reduced to grade W, of 1,000,000 or more. Any sample that had a plate count of 5,100,000 could be expected to reduce to grade 5 or beyond within an hour.

Due to the great amount of overlapping shown in the table, it does not seem feasible to make any further predictions regarding correlation. Neither does it seem possible to assign various grades such as those used with methylene blue, and relate them to any particular color after one hour's incubation. While this has been done by some investigators, there is no basis for it in our results. It will be noted that grades 1 to 4 include samples with plate counts ranging from under 75,000 to 5,000,000. Grades 5 and 6 include the whole range of plate counts, and grades 7 and 8 include counts from 76,000 to over 5,100,000. The danger of attempting fine distinctions in correlation is readily apparent.

Table 2 shows the number of samples of each plate count group falling into the resazurin groups for the ten minute test. Study of this table will show that the ten minute test is of very little more value than the one hour test. All samples reaching grade 4 or beyond could be assumed safely to be of poor quality. The number of low count milks tending to confuse the picture in grade 3 is not large, and most of these, too, are of poor quality. However, it will be noted that the largest percentage of high count milks falls into grades 1 and 2 rather than into the poorer grades. In the 1,100,000 to 5,000,000 group, over a third of the samples

Table 2

Relation Between Resazurin Ten Minute Test

and Plate Count

Standard Plate Count	Resazurin Grades									
	1	2	3	4	5	6	7	8	W	Totals
10,000 and under	25	2	1	-	-	-	-	-	-	28
11,000 - 50,000	81	9	-	-	-	-	-	-	-	90
51,000 - 100,000	28	3	1	-	-	-	-	-	-	32
110,000 - 250,000	16	8	2	-	-	-	-	-	-	26
260,000 - 500,000	32	13	2	-	-	-	-	-	-	47
510,000 - 1,000,000	26	15	4	-	-	-	-	-	-	45
1,100,000-5,000,000	29	31	11	-	-	-	-	-	-	83
5,100,000 and over	4	6	5	4	9	8	6	2	9	53
							Total Samples			404

failed to show any reduction whatsoever; and even a bacterial content of over 5,100,000 was insufficient to initiate reduction in all the samples of this latter group.

In attempting to explain this poor correlation, the factors affecting both tests will be briefly considered. Johns (24) has pointed out the larger size of the sample, the sensitivity to growth phase, and the lack of influence by clumping, as features of the resazurin test that were advantages. To these may be added the difference in reducing activity of various organisms, the effect of body cells, the effect of the pH of the milk, and the effect of the reducing system of the milk itself, apart from the bacterial reducing system. The interrelationship of the various reducing systems in a milk sample is at present ill-defined, but may affect the test significantly.

The plate count may correlate poorly with a reductase test since one colony may represent one organism or a clump of a hundred. The standard medium may fail to support certain organisms and the temperature is not the optimum one for all organisms in the milk. The plate count samples a smaller amount of milk than the resazurin test and is insensitive to growth phase. The plate count measures quantity, the reductase test biological activity. It appears from the above list that it is perhaps futile to even attempt a correlation between two tests; however, a new test must be compared to a standard of some kind if its value is to be assessed.

While the resazurin test may possess merits which can be demonstrated by other methods of study, these are not apparent in attempting to compare

it with the standard plate count. Tests read over a longer period of time, such as the triple reading test of Johns and Howson (29) are claimed to give more accurate results. We have refrained from lengthening the reading time since shorter reading time is one of the chief advantages of resazurin over methylene blue, and as Davis (10) has observed, lengthening the reading time eliminates this advantage to a large extent. It is doubtful whether the British "routine" resazurin test (52) could be successfully carried out on this continent. The compensating scale used in this test makes no provision for extremes of temperature which occur annually in many sections of America. The use of room temperature tests which has been reported from Britain would also be precluded in America because of the wide range of room temperatures which prevails during the year in many laboratories.

B. Resazurin-Rennet Test

The relation between the resazurin rennet test and the other mastitis tests which were used on the quarter samples is shown in Table 3. Where letters appear after a sample number they indicate that those quarters belong to the same cow, the number representing the cow and the letters the quarters. The readings of the brom cresol purple test (BCP +) indicate the intensity of the purple color reaction. The readings of the catalase test are expressed in terms of ml. of oxygen. Bacterial counts are not included, as bacteria were only rarely observed in Breed smears from aseptically drawn, unincubated quarter samples. The resazurin results

Table 3

Resazurin-Rennet Test
Quarter Samples
Correlation with other Tests

Sample	Strip Cup	BCP	Hotis	Catalase	Resazurin 10 min	Resazurin 60	Rennet Time	Udder Cells	Organism Isolated
1	+	+	-	6	2	6	45	3,500,000	-
2	+	++	-	6	1	7	-	t.n.t.c.*	-
3	-	+	-	9	1	1	-	600,000	-
4	-	+	-	8	2	6	-	15,500,000	S. aureus
5a	-	-	-	1	1	2	30	200,000	-
5b	+	+++	-	10	7	W	-	23,000,000	G- rod
5c	-	-	-	1	1	2	30	150,000	Micrc.
5d	-	+	-	10	1	1	-	6,600,000	-
6	-	+	-	6	1	4	45	6,200,000	-
7a	+	+	-	9	3	8	-	6,300,000	S. uberis
7b	-	-	-	1	1	1	-	none	Micrococ.
7c	+	++	-	10	1	2	-	4,600,000	S. aureus
7d	+	-	-	0	1	2	60	none	S. aureus
8a	-	+	-	2	1	5	60	1,500,000	-
8b	-	-	-	0	1	1	45	300,000	-
9	+	+	-	5	1	3	-	1,500,000	S. aureus
10	+	±	-	2	1	2	45	500,000	-
11	+	-	-	0	1	2	60	150,000	-

(continued)

Table 3 (continued)

Sample	Strip Cup	BCP	Hotis	Catalase	Resazurin 10 min	Resazurin 60	Rennet Time	Udder Cells	Organism Isolated
12	-	-	-	0	1	2	30	none	-
13	-	+	-	15	2	6	105	2,200,000	S. aureus
14	-	+	-	5	1	1	60	290,000	S. aureus
15a	+	+	-	13	2	6	30	3,800,000	S. uberis
15b	-	-	-	1	1	1	30	120,000	-
16	-	+	-	7	2	5	45	1,700,000	S. aureus
17a	-	+	-	6	1	2	30	600,000	E. coli
17b	-	+	-	1	1	2	30	none	-
18	+	±	-	1	1	2	45	180,000	S. aureus
19	-	-	-	4	1	2	45	820,000	S. aureus
20	-	+	-	3	1	3	45	500,000	-
21	-	+	-	9	2	5	45	3,100,000	-
22a	-	+	+	6	1	4	30	2,100,000	S. agal.
22b	-	+	+	3	1	3	30	1,100,000	S. agal.
23	-	+	+	5	1	2	75	960,000	S. agal.

*t.n.t.c. = too numerous to count

show the Munsell color grade attained after 10 minutes and 60 minutes respectively. The rennet coagulation time is expressed in minutes. A negative sign (-) indicates that no coagulation took place within 2 hours.

Most of the quarter samples submitted to the resazurin-rennet test were taken because they appeared positive either on the strip cup, the brom cresol purple test, or on both. The subsequent correlation with these and the Hotis and catalase tests are shown in Table 3. The reducing influence of the udder cells from the infected quarters of cow no. 5 stands out quite clearly against the slow reduction (and fast clotting) of the milk from the two normal quarters. However, in cow no. 7 there is a rennet false positive in the apparently normal "b" quarter, and a resazurin false negative in the "c" quarter which was high in udder cells and harbored Staph. aureus infection. False negatives also occur in samples 7a, 9, 14, 17a, 18, 19 and 23 in the presence of pathogenic organisms.

The failure of resazurin to give a satisfactory correlation with the other tests is not in itself an indictment, since each individual test is subject to error. However, its failure to indicate positively against positive results on a combination of the other tests, and against positive cultural results, reveals that it is of little value in the diagnosis of mastitis. The number of false negative resazurin tests is not of great statistical significance in view of the small number of samples presented; however, the number of false negatives is disturbingly large, inasmuch as 50 per cent of the samples with positive cultural results gave negative resazurin tests. This percentage does not include the samples which appeared markedly abnormal but negative culturally.

The failure of the mastitis organisms, though present, to influence the test is undoubtedly due to their small numbers. Theoretically, it might be feasible to incubate the samples until the pathogenic bacterial content rose to the point where it would affect the test. However, this period of incubation would greatly reduce the activity of the udder cells, and also increase the non-pathogenic bacterial population, making the results very confusing from a diagnostic standpoint. The latter point is of practical importance. Many of the samples reaching the Veterinary Hygiene Laboratory at Iowa State College contain numbers of saphrophytic bacteria as contaminants. These organisms almost invariably overgrow any pathogens present if incubation is attempted.

The resazurin-rennet test was also performed on two composite herd samples to determine its value in detecting mastitis milk in a producer's supply. Two herds were checked by the routine mastitis tests shown in Table 3; however, only quarters from which pathogenic organisms were isolated were considered infected. The incidence of subclinical udder infection, as determined by cultural examination, is shown in Table 4.

Table 4

Extent of Mastitis Infection in Two Dairy Herds

Herd	No. of Cows	No. of Qtrs.	Normal Qtrs.	Atrophied and non-lactating	Infected Quarters
Herd A	17	68	65	--	3 (4.4%)
Herd B	19	76	59	5	12 (15.8%)

The results of the resazurin-rennet test on three consecutive days for

the bulk samples from these herds is shown in Table 5. The cell count is the arithmetic average of the counts for the three days. The numbers in the body of the table are the Munsell color grades at the incubation times shown.

Table 5

Resazurin-Rennet Test of Mixed Herd Samples

Herd	Breed Cell Count	Clotting Time	Resazurin Grades ¼hr	½hr	3/4hr	1hr	1½hr	2hr
Herd A	275,000	30 min.	1	2	2	3	4	7
		30 min.	2	3	5	6	7	8
		45 min.	1	2	2	4	5	6
Herd B	360,000	30 min.	1	1	1	1	1	2
		30 min.	1	1	1	2	3	3
		30 min.	1	1	2	2	3	3

Table 4 shows that herd A had an incidence of infection of 4.4% of its lactating quarters, while the incidence in herd B was 15.8%. Due to prompt and efficient cooling on the B farm, the bacterial population in this milk was kept very low. It is obvious that the mastitis infection itself was not able to cause any marked reduction or clotting abnormality in the presence of dilution with the milk from the normal quarters. On the other hand, rather careless production methods on the A farm resulted in a high bacterial content which gave much faster reduction, even though the incidence of mastitis infection was little more than a quarter of that in the B herd.

It is true that microscopic examination would reveal that the reduction in the A milk was being caused by contaminating bacteria; but this

would not enhance the value of the resazurin-rennet test as a mastitis indicator in view of the essentially negative results obtained from herd B. A careful observation of the character of the reduction might also indicate whether the reduction was due mainly to udder cells or to bacteria. However, this too, would not obviate the false negative result obtained with herd B. In a test of this sort a false negative is much more serious than a false positive, because a positive would be followed by examinations to detect the individual infections, whereas a negative result would not be followed up.

C. Effect of Udder Cells

The influence of udder cells on the resazurin test is shown in Tables 6 and 7. The numbers in the tables are the Munsell color grades at the incubation times shown. Table 6 shows that removing a portion of the cells by centrifuging resulted in less active reduction, as compared to the uncentrifuged milk. Table 7 illustrates that the addition of the centrifuge sediment from mastitis milk to sterile milk resulted in more rapid reduction.

These results are in accordance with those of Johns (26) who found that the addition of centrifuge sediment to milk hastened color change in the resazurin test. Thomas (50) reported similar findings. The original suggestion of Strynadka and Thornton (49) regarding an increased secretion of reducing substances in mastitis milk must still be considered, pending further elucidation of the biochemistry of resazurin reduction in milk. It appears, however, that if such substances are present, they are

Table 6

Action of Udder Cells in Reducing Resazurin

	Mastitis Milk							Centrifuged Mastitis Milk						
Sample	1/4*	1/2	3/4	1	11/2	2	21/2	1/4	1/2	3/4	1	11/2	2	21/2
1	2	3	4	5	6	6	6	1	1	2	2	2	2	3
2	3	3	4	5	6	6	7	2	3	4	5	6	7	7
3	1	2	2	2	2	3	3	1	1	1	1	1	1	1
4	1	1	1	2	2	2	2	1	1	1	1	1	1	1
5	1	2	2	2	2	3	3	1	1	1	1	1	1	1
6	1	2	2	3	4	4	4	1	1	1	1	1	1	2

* Incubation time in hours

Table 7

Action of Udder Cells in Reducing Resazurin

	Sterile Milk							Milk and Cells						
Sample	1/4	1/2	3/4	1	11/2	2	21/2	1/4	1/2	3/4	1	11/2	2	21/2
1	1	1	1	1	1	1	1	2	3	4	5	5	6	6
2	1	1	1	1	1	1	1	1	1	2	2	2	2	2
3	1	1	1	1	1	1	1	1	2	2	2	2	2	2
4	1	1	1	1	1	1	1	1	2	2	2	2	3	3
5	1	1	1	1	1	1	1	1	2	2	3	3	3	3
6	1	1	1	1	1	1	1	2	3	3	4	4	4	5

associated with fresh, unwashed udder cells, since either washing or ageing of the cells greatly retards the reducing activity of the milk. Strynadka and Thornton used washed leucocytes obtained from blood and peritoneal exudate, and when they were unable to shorten resazurin reduction time by adding these, they concluded that leucocytes did not affect reduction. Not only did they fail to consider the weakening effect on the cells of washing, but also the fact that the various leucocytes were present in a different proportions in mastitis milk and in the blood stream. In addition mastitis milk usually contains epithelial debris as well as leucocytes. Since the effects of each individual type of cell on resazurin are not known, it is possible that the predominance of polymorphonuclear leucocytes in mastitis milk accounts for the discrepancy between the results of Strynadka and Thornton and those of the other investigators.

While not a formal part of this study, opportunity was taken on several occasions to observe the effect of storage on the reducing power of udder cells. Mastitis milk samples were divided to give two 10 ml. samples, one of which was tested immediately and the other after 24 hours refrigerator storage. It was noted that much of the reducing power of udder cells tended to disappear after 24 hours storage. This was most evident in those samples which had shown rapid reduction when tested in the fresh state.

V. DISCUSSION

The milk sanitarian is interested in a test which will detect raw milk that is abnormal, and hygienically or aesthetically undesirable, and enable its degrading or complete diversion from fluid milk processing. To attain this end, he must first define the qualities which he considers undesirable; the value of any test then lies in the efficiency with which it detects these qualities.

The tendency in the past has been to consider milk quality as synonymous with bacterial content; although butterfat, adulteration, acidity and sediment tests have been carried out as well. Generally speaking, however, quality in unadulterated milk has meant bacterial quality. While this concept held sway, controversy revolved around which method measured the bacterial content more effectively--the reduction test or one of the counting methods. A vast amount of work was done both here and abroad in comparing methylene blue reduction with plating and direct counting. In view of the many variable factors causing error in the three methods, it is not surprising that much of this work led to inconclusive results. However, a more or less satisfactory relation was finally established with the counting methods, and the methylene blue test adopted officially by the A.P.H.A. As pointed out in "Standard Methods", the amount of space and equipment needed for methylene blue tests is small, and the method is especially suited to grading raw milk samples quickly. It is useful in small plants with meager laboratory facilities.

With the development of the resazurin tests, it soon became evident that resazurin was sensitive to various influences which did not appear to affect methylene blue. A spirited controversy existed for a time as to which was a better indicator of milk quality, still in the old sense. The results of careful experimental work showed that methylene blue was a more accurate indicator of bacterial content than resazurin, when both dyes were compared with the counting methods.

Ramsdell pointed out that the other factors affecting the resazurin test, while not an index of bacterial content, were nevertheless an indication of undesirable milk. He contended that mastitis milk, colostrum and late lactation milk have no place in market milk, and cited the ability of resazurin to detect their presence as an advantage. Thus he introduced a wider concept of the term "quality" as applied to milk, and one which sanitarians, with a few notable exceptions, have been willing to accept.

Subsequent workers, therefore, attempted to determine how accurately resazurin could detect the above abnormalities. That they are undesirable was conceded, whether resazurin could detect them became the subject of investigation. Frayer (19) pointed out that resazurin underwent changes in milk not necessarily connected with poor quality. Watts and Stirling (58) pointed out that the dilution factor prevented the detection of mastitis milk in bulk samples. The action of washing and aging on udder cells was also mentioned by Johns (26) and Davis (10). Most of this work showed that although cells would indeed cause a characteristic reduction of resazurin, that the action of cells following dilution with normal milk, and after the usual period of storage, (12-24 hours), had no appreciable

influence on the test.

Thornton et al (54) ignored the new definition of quality, and assessed the value of resazurin purely on its ability as a quantitative bacterial indicator. From this standpoint its sensitivity to non-bacterial factors in abnormal milk is obviously a disadvantage. However, Johns (26) pointed out the fallacy of such reasoning inasmuch as a physiologically or pathologically abnormal milk was not a first-grade product even though its bacterial content might be low. Work carried out on mastitis milk in the Veterinary Hygiene Laboratory at Iowa State College indicates that the bacterial content of such milk is often quite low. In some instances the milk may be visibly pathological and yield no bacteria on culture. Johns also mentioned that such milk is of significance where homogenization is to be carried out. This point, while of no public health significance, is of considerable importance to the dairyman.

The only value that appears to lie in the one hour test is in separating the very bad samples, with counts of 1,000,000 and over from the bad, fair and good samples. The fact that all the 75,000 and under samples are grade 6 or better is of little significance, since 153 samples with higher plate counts (representing all but the last group) are also between grades 1 and 6. The advantages of a dye reduction test on the basis of cost, simplicity and convenience, as set forth by Johns (25) are offset by the unreliability of the one hour test.

The ten minute test is probably of value for the rapid detection of the worst quality milk, especially in the summer, when high count milk is prevalent in many supplies. Neither of the tests appears to form a

reliable basis for grading the better quality milks.

The results of the resazurin-rennet test on quarter samples do not illustrate that this test is of any particular value in detecting mastitis as reported by Davis (11). Neither do they illustrate the ability of resazurin to detect mastitis streptococci and pathogenic staphylococci, reported by Russell et al (46). The 50 per cent false negatives obtained in this study agrees with the findings of Watts and Stirling (58), who reported a false negative range of 30 to 88 per cent with various modifications of the resazurin test. They were unable to devise any test which would give less than 30 per cent false negatives, and concluded that resazurin reduction was of very limited value in the routine diagnosis of mastitis.

The resazurin-rennet test failed to detect the presence of mastitis milk in a herd in which udder infection involved over 15 per cent of the lactating quarters. It gave misleading results on composite milk from a second herd where the incidence of infection was only 4.4 per cent. These results confirm the contention of Watts and Stirling (58) that the resazurin test is of little value in the detection of mastitis in bulk milks. It should be remembered, of course, that the statistical significance of two herd checks is not great, and further work along these lines is required in order to establish a definite trend. Such work should be done both with small herds, where the incidence of infection is usually low, and the dilution factor small; and with large herds, where a high incidence often prevails, but the dilution factor is large.

The resazurin test should not be discarded because of the presently

unexplained factors which tend to influence the relation between bacterial content and reduction time. While admitting the presence of these factors, one must keep in mind that the counting methods, too, have their inaccuracies. Geiger's contention, that the reduction tests are wrong in theory, seems somewhat extreme at this time (20). The basis of judgment should be relative rather than absolute. In other words, is resazurin as accurate in its sphere of activity as the counting methods are in theirs? If one decides only that it is no less accurate, then its advantages of convenience, simplicity and low cost must outweigh those of the counting methods (25). On the other hand, accuracy must be the determining factor and should not be sacrificed to the above advantages.

Thornton et al (54) pointed out in 1941 that there was very little information available on the chemistry of the reduction of resazurin. There has been little added to the published information on this aspect of the test since that time. The problems of synthesis and standardization have been solved, and a number of variables affecting the test are known. If the results are to be of scientific value, further attempts to assess the value of the resazurin test must await elucidation of the chemical fundamentals involved.

VI. CONCLUSIONS

1. The standard resazurin tablets give a more satisfactory solution than did the powder which was formerly used.

2. A satisfactory technic for the preparation of resazurin solution is described.

3. A modified comparator which facilitates the reading of the resazurin test is described.

4. The addition of rennet to the resazurin solution in the amount used does not affect the resazurin reduction rate.

5. Compared with the Standard Plate Count, the one hour resazurin test does not appear to be a satisfactory test for grading milk of good to fair quality.

6. Compared with the Standard Plate Count, the ten minute resazurin test does not appear to be a satisfactory test for grading milk. It shows promise as a rapid test for separating very poor quality milk from poor, fair and good milk.

7. The udder cells present in freshly drawn mastitis fore-milk play a definite part in resazurin reduction, and their reducing ability tends to disappear upon storage.

8. The resazurin-rennet test appears to be unsatisfactory as a diagnostic test for mastitis when applied to quarter samples.

9. The resazurin-rennet test appears to be of slight value in detecting the presence of mastitis milk in composite herd samples.

VII. LITERATURE CITED

1. Abele, C.A. The methlene blue reduction time as a means of estimating the bacterial content of milk to determine its suitability for pasteurization, or as a basis for grading. Jour. Milk Tech. 8:67. 1945.

2. American Public Health Association. Standard methods for the examination of dairy products. 8th ed. p. 66. New York. 1941.

3. Ayers, J.C. Ames, Ia. Information on bacterial content of foods and resazurin reduction. Private communication. 1946.

4. Baker, W. et al. The synthesis and standardization of sodium resazurate for testing the hygienic quality of milk. Biochem. Jour. 36, nos. 1-2:i-ii. 1942.

5. Barkworth, H. Technology. Dairy Industries 9:409-410. 1944.

6. _____ and Jones, G.E. Statistical control of color reading in the resazurin test. Proc. of the Society of Agr. Bact. 1944:40-42. 1944.

7. Barret, D.W. et al. The resazurin test--its use and practicability as applied to the quality control of milk. Jour. Dairy Sci. 20:705-709. 1937.

8. Collins, M.A. et al. Application of the resazurin test in determining quality of raw milk and cream. Jour. Milk Tech. 2:236-244. 1939.

9. Davis, J.G. The resazurin-rennet test for the commercial control of milk supplies. Dairy Industries 4:421-423. 1939.

10. _____ The resazurin test--a review of recent work. Dairy Industries 5:19-21. 1940.

11. _____ Bacteriological methods for the large scale detection of mastitis. Veterinary Record 54:145-147. 1942.

12. _____ and Jones, V.E. The differentiation of cells from bacteria in the resazurin test. Proc. of the Society of Agr. Bact. 1944:44-46. 1944.

13. _____ and Thiel, C.C. Observations on the resazurin and methylene blue tests. Proc. of the Society of Agr. Bact. 1939:35-36. 1939.

14. _____ and Thomas, S.B. A comparator for the resazurin test. Dairy Industries 5:244-246. 1940.

15. _____ et al. The resazurin and repid resazurin tests--their relation to other bacteriological tests and mastitis. Dairy Industries 8:29. 1943.

16. Fabian, F.W. Laboratory experiments in dairy bacteriology. p. 5. Minneapolis. Burgess Publishing Co. 1945.

17. Frayer, J.M. The resazurin test, a preliminary study. Vermont Agr. Expt. Sta. Bull. 435:12-16. 1938.

18. _____ The dissolved gases in milk and dye reduction. Vermont Agr. Expt. Sta. Bull. 461:28-30. 1940.

19. _____ Dye reduction in milk related to Eh, pH and dissolved gases. Vermont Agr. Expt. Sta. Bull. 498:32. 1942.

20. Geiger, J.C. et al. Safe milk supplies of cities and the public health laboratory. Milk Plant Monthly 35, no. 5:71. May 1946.

21. Golding, N.S. and Jorgensen, S.I. A correlation of the resazurin grade with the standard plate count of raw milk. Jour. Milk Tech. 8:191-194. 1945.

22. Jackson, C.J. Technique for obtaining anaerobic milk, with some observations on its CO_2 content. Jour. Dairy Res. 7:25-26. 1936.

23. Johns, C.K. Concerning the accuracy of the methylene blue reduction test. Jour. Dairy Sci. 21:227-237. 1938.

24. _____ The place of the methylene blue and resazurin reduction tests in a milk control program. Amer. Jour. of Public Health. 29:239-241. 1939.

25. _____ Tests for Milk Quality. Canadian Public Health Jour. 32:27-31. 1941.

26. _____ Recent Canadian research on the resazurin test. Proc. International Assn. of Milk Dealers 34:256-265. 1942.

27. _____ Dye concentrations in resazurin tablets. Amer. Jour. of Public Health 34:955-956. 1944.

28. _____ and Howson, R.K. Potentiometric studies with resazurin and methylene blue in milk. Jour. Dairy Sci. 23:302. 1940.

29. _____ and Howson, R.K. A modified resazurin test for the more accurate estimation of milk quality. Jour. Milk Tech. 3:323-324. 1940.

30. Keenan, J.A. et al. The resazurin test--preliminary studies on its practicabilities and possibilities. Jour. Milk Tech. 1:22-23. 1937.

31. Little, Lawrence. Comparative studies upon the methylene blue and resazurin tests. Jour. Milk Tech. 3:274-279. 1940.

32. Moldavan, Andrew. The resazurin test. Milk Plant Monthly 27, no. 10:58-60. Oct. 1938.

33. Morgan, G.F.V. and M'Kenzie, D.A. The use of the resazurin test as a rapid test for the indication of mastitis. Proc. of the Society of Agr. Bact. 1943:64-66. 1943.

34. Mundinger, E. and Wolf, E. Die Usurufin Probe, eine verbesserte Reduktaseprobe. Molkerei Zeitung 47:171-173. 1933.

35. Morris, C.S. and Edwards, M. Effect of high cell content on the resazurin and methylene blue tests. Dairy Industries 10:92. 1944.

36. Nelson, F.E. A note on the suitability of various lots of dye for use in the resazurin reduction test. Jour. Dairy Sci. 22:335-336. 1939.

37. _____ and Foltz, V.D. Studies on resazurin as an indicator of the quality of milk. Reports of the Kansas Agr. Expt. Sta. 1936/38:89-90. 1938.

38. Packer, R.A. Ames, Ia. Information on bacterial catalase production in milk. Private communication. 1946.

39. Pesch, K.L. and Simmert, H. Untersuchungen über den frischeitszustand der Milch. Milchwirtchaftliche Forschungen 8:568-575. 1929.

40. Phillips, G.M. and Soulides, D. The variability of the routine resazurin test. Proc. of the Society of Agr. Bact. 1943:50-52. 1943.

41. Proctor, B.E. and Greenlee, D.G. Correlation of resazurin reduction rates and bacterial plate counts as indices of the bacterial condition of fresh and frozen foods. Food Res. 4:441-446. 1939.

42. Provan, A.L. and Rowlands, A. Testing pasteurized milk--the use of dye reduction tests. Dairy Industries 8:67-70. 1943.

43. Ramsdell, G.A. The resazurin test for sanitary condition of milk. Milk Dealer, 27, no. 11:90. Nov. 1937.

44. _____ et al. Investigation of resazurin as an indicator of the sanitary condition of milk. Jour. Dairy Sci. 18:705-717. 1935.

45. Rowlands, A. Dairy laboratory control. Dairy Industries 9:170. 1944.

46. Russell, R.R. et al. The resazurin test for detecting mastitis. New Zealand Jour. of Sci. and Tech. 21:161A-167A. 1939.

47. Schacht, F.L. and Nichols, R.E. Studies of the resazurin-rennet test. Jour. Milk Tech. 4:281-284. 1941.

48. Skar, Olav. Omkring Reduktaseprøven. Skandinavisk Veterinär-Tidskrift 32:47-48. 1942.

49. Strynadka, N.J. and Thornton, H.R. Leucocytes and the methylene blue reduction test. Jour. Dairy Sci. 21:568. 1938.

50. Thomas, S.B. Further investigations on the resazurin test. Welsh Jour. of Agr. 27:117-130. 1943.

51. _____ and Bowie, D.A. The use of the resazurin test as a rapid test for the indication of mastitis. Proc. of the Society of Agr. Bact. 1943:64-66. 1943.

52. _____ et al. The routine resazurin test. Welsh Jour. of Agr. 28:81-95. 1945.

53. _____ and Probert, D. The cellular content of milk. Part IV. Dairy Industries 6:295-297. 1941.

54. Thornton, H.R. et al. The reduction of resazurin in milk and aqueous solutions. Canadian Jour. Res. 19, sec. B:39-48. 1941.

55. Twigg, R.S. Oxidation reduction aspects of resazurin. Nature 155:401-402. 1945.

56. Waldbauer, Olga. The resazurin reductase test. Kisérletügyi Közleményeki 34:128-132. 1931. (Original not seen, abstracted in Chem. Abst. 26:2797.) 1932.

57. Warner, J.N. The use of resazurin in determining the bacterial quality of milk and ice cream. Unpublished M.S. thesis. Ames, Ia. Iowa State College Library. 1937.

58. Watts, P.S. and Stirling, A.C. Mastitis and the resazurin test. Veterinary Record 56:83-85. 1944.

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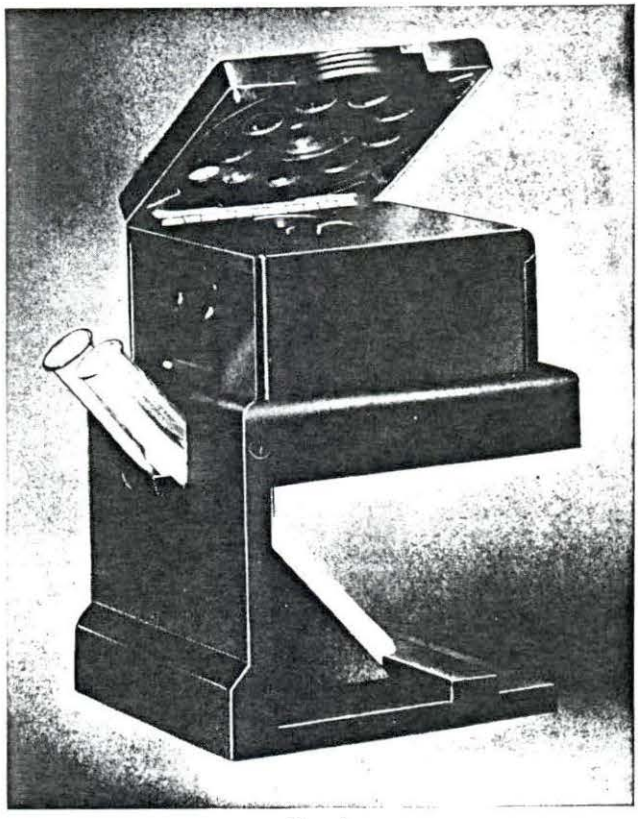

FIG. I.

Lovibond Comparator

(Open, showing revolving color discs)

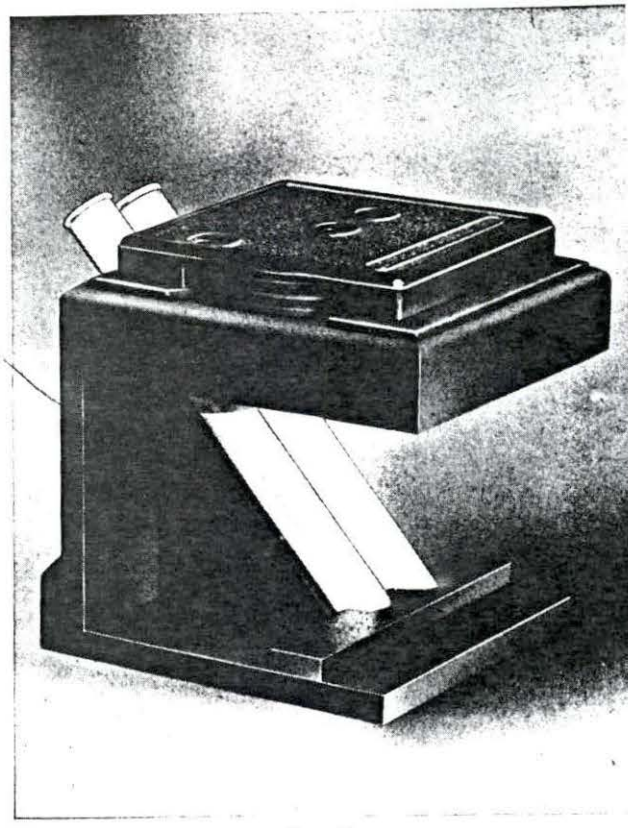

FIG. II.

Lovibond Comparator

(Closed for reading)

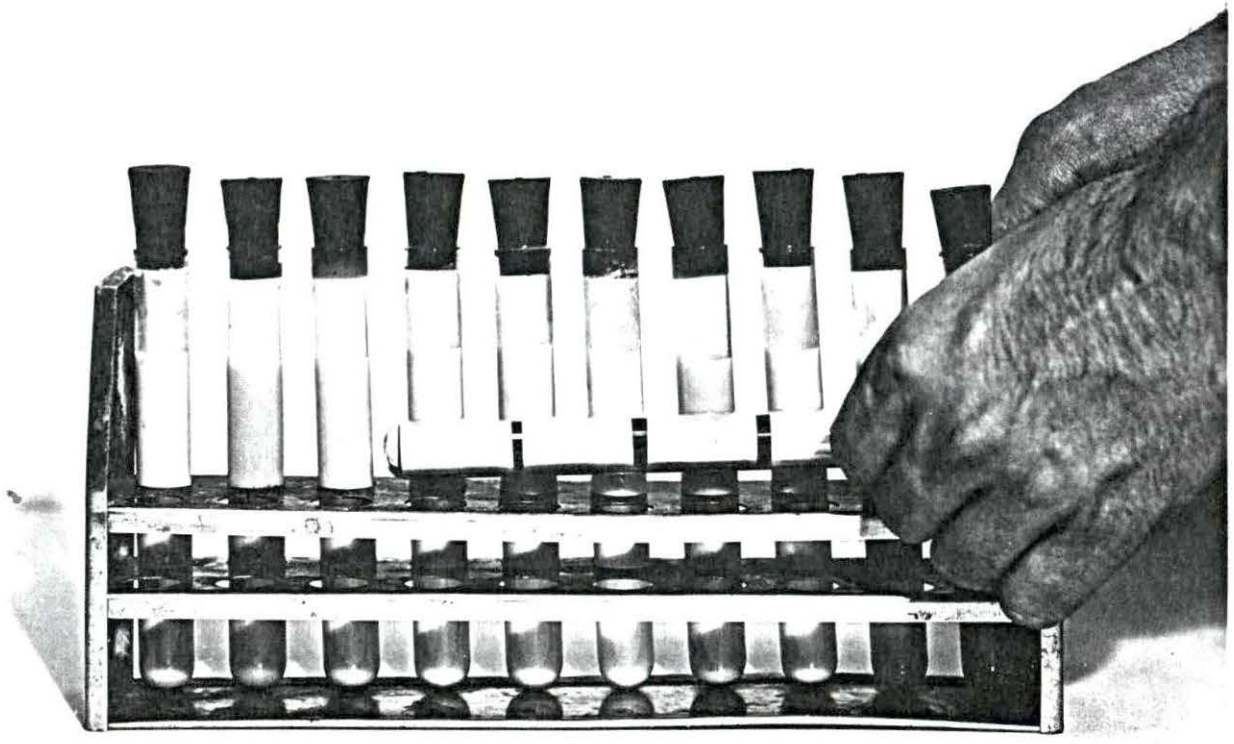

Figure 3. Munsell Color Grader

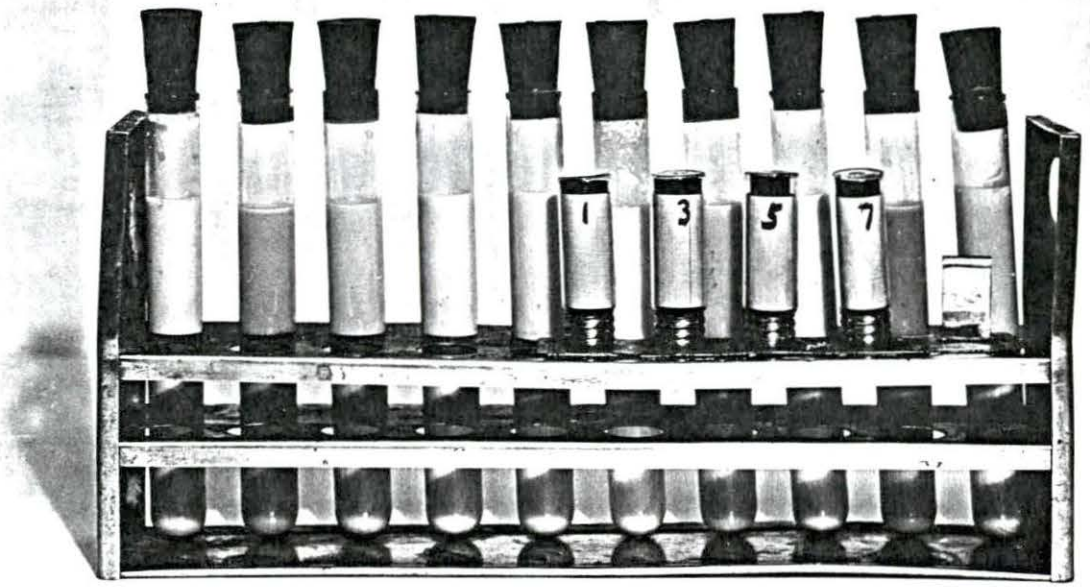

Figure 4. Modified Munsell Grader

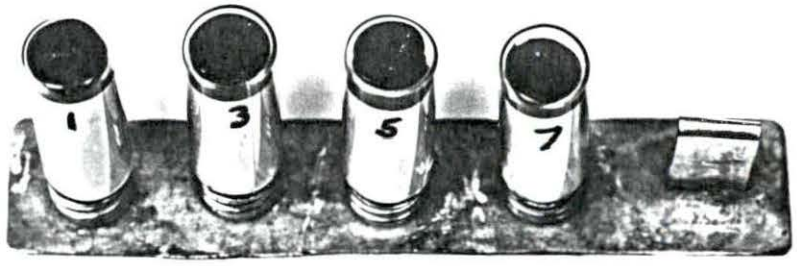

Figure 5. Munsell Color Grader (above), and Modified Munsell Grader